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SOLUBLE HETERODIMERIC T CELL RECEPTORS AND THEIR ANTIBODIES

Abstract:

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(57) Abstract

Heterodimeric T cell receptor proteins, comprising $\alpha$ and $\beta$ subunits joined by at least one disulfide bond, are disclosed. The $\alpha$ and $\beta$ subunits are generated as chimeric polypeptides, utilizing $\zeta$ chains or constant regions of IgG1 as chimeric partners. Also disclosed are soluble heterodimeric T cell recpetor molecules with a conformation essentially indistinguishable from that appearing on the surface of T cells. The invention also concerns DNA encoding the heterodimeric TCR, transfer vectors comprising DNA encoding the heterodimeric TCR, and host cells containing such transfer vectors. In addition, the invention pertains to various uses of heterodimeric TCR. The proteins can be used in molecular assays designed to measure their binding to ligands, including MHC/HLA-peptide antigen complexes or TCR-specific antibodies. Such assays are useful for the detection of agents that block the TCR-ligand interaction. The heterodimeric TCR can also be used to immunize animals, including humans, to produce TCR-specific antibodies. In addition, either in their native or denatured conformation, the proteins can be used to vaccinate animals, including humans, in order to suppress the immune response of T cells bearing TCR that share antigenic epitopes with the vaccinating protein.

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# SOLUBLE HETERODIMERIC T CELL RECEPTORS AND THEIR ANTIBODIES

## Background of the Invention

The T cell receptor (TCR) is a clonally expressed cell surface protein of T lymphocytes which mediates recognition of foreign antigens. It is composed of six polypeptide chains, two of which form a heterodimer and are unique to any given clonal T cell line. Four polypeptides ($\alpha$, $\beta$, $\gamma$, $\delta$) form two different heterodimers ($\alpha$:$\beta$ and $\gamma$:$\delta$); the $\gamma$:$\delta$ heterodimer appears earlier than the $\alpha$:$\beta$ heterodimer in the development of an organism (Davis, M. M. and P. J. Bjorkman, *Nature 334*:395-402 (1988)). The amino terminal half of the $\alpha$ and $\beta$ (or $\gamma$ and $\delta$ depending on the T cell subtype) chains which comprise the TCR are known as the variable (V) regions because the unique specificity of the TCR is a reflection of the marked amino acid sequence diversity of these regions. This sequence diversity determines the specificity of the TCR, enabling recognition of a vast array of protein fragments, or epitopes, presented by the "restricting element", the major histocompatibility complex (MHC) (known in humans as the HLA complex) class I and class II proteins (Germain, R.N., *Cell 76*:287-299 (1994)). Recognition by the TCR of antigen in the context of MHC (or HLA) molecules triggers T cell activation, thus initiating the immune response.

The sequences of the TCR $\alpha$- and $\beta$-chain variable regions are encoded by gene segments that undergo somatic recombination to form complete transcriptional units during T cell development (Davis, M.M. and P. Bjorkman, *Nature 334*:395-402 (1988)). Because rearrangements of the V and J segments of the $\alpha$-chain family and the V, D, and J segments of the $\beta$-chain family occur independently in each developing T cell, the TCR repertoire of antigen-binding

specificities is expressed clonally. This has been demonstrated by the observation that the sequences of the functionally rearranged TCR genes from independently derived T cell clones encode TCR α- and β-chains with different primary amino acid sequences (see, for example, Fink, P.J. *et al.*, *Nature 321*:219-226 (1986)).

Many groups have tried different approaches for producing soluble paired variable regions of the α/β TCR: (i) as variable regions connected by a polypeptide linker to create single chain (sc) TCR molecules; (ii) as fusions with immunoglobulin kappa light chains; and (iii) as phosphotidylinositol-linked heterodimers on the surface of cells in tissue culture.

The single chain TCR (scTCR) approach outlined by Novotny *et al.* (*PNAS USA 88*:8646-8650 (1991)) relies on expression of the scTCR α/β in *E. coli*. This expression system offers efficient production of protein in high yields; however, much of the bacterially-derived scTCR is aggregated, improperly folded and insoluble. Refolding of purified and denatured recombinant proteins is often an inefficient process because the denatured scTCR is highly insoluble and prone to aggregation or precipitation when undergoing refolding. Moreover, the apparent low solubility of the scTCR as expressed in bacteria in aqueous solvents further reduces the yield following renaturation.

There have been many attempts to express TCR α- and β-chains in eukaryotic cells (Traunecker, A., *et al.*, *Immunol. Today 10*:29 (1989). When the genes were initially cloned into appropriate expression vectors and transfected into cultured mammalian cells, expression of TCR α/β heterodimers could not be obtained in the absence of coexpression of γ, δ, ε, and ζ chains; that is, the other members of the group of proteins that together form the CD3 complex. If the α- and β-chains were not assembled into a CD3 complex, the protein was degraded in the endoplasmic

reticulum (Wileman *et al.*, *Cell Regulation* 1:907-919 (1990)). It was later determined that a signal for degradation resided in the transmembrane region of the TCR α and β polypeptide chains (Wileman *et al.*, *Cell Regulation* 1:907-919 (1990); Wileman *et al.*, *J. Cell Biol. 110*:973-86 (1990); Bonnifacino *et al.*, *Science 247*:79-84 (1990); Bonnifacino *et al.*, *Cell 63*:503 (1990); Shin *et al.*, *Science 259*:1901 (1993)).

It has also been shown that both α and β chain extracellular domains can be synthesized as soluble chimeric molecules with carboxy-termini derived from immunoglobulin molecules (Gregoire, C. *et al. Proc. Natl. Acad. Sci. USA 88*:8077-8081 (1991); Gascoigne, N.R.J. *et al.*, *Proc. Natl. Acad. Sci. USA 84*:2936-2941 (1987); Weber, S. *et al.*, *Nature 256*:793-796 (1992)).

Phosphatidyl inositol membrane anchored α/β TCR heterodimers have been produced on the surface of CHO cells, and enzymatically released from the cell surface by phospholipase C treatment ((Lin, A.Y. *et al.*, *Science* 249:677 (1990); Slanetz A.E. and Bothwell, A.L.M., *European Journal of Imm.* 21:179-183 (1991)); however, small amounts of soluble TCR were produced, and the method is not practical for the production of milligram quantities.

## Summary of the Invention

The present invention concerns a polypeptide molecule comprising a heterodimeric T cell receptor (TCR) molecule containing a β subunit connected by disulfide bonds to an α subunit of the TCR. The α and β subunits are generated as separate chimeric polypeptides, comprising an α segment or a β segment in conjunction with either a ζ chain or an immunoglobulin constant region as a chimeric partner. Alternatively, the α and β subunits can be generated as non-chimeric polypeptides comprising an α or a β segment. The chimeric partners can be removed from the subunits

through enzymatic cleavage. Heterodimeric TCR from which the chimeric partners have been removed or are absent are soluble dual chain (dc) TCR. The heterodimeric TCR of the current invention is a TCR protein, soluble in aqueous solvents containing simple buffers, with a conformation that is functionally indistinguishable, based upon reactivity with clonotype-specific antibodies, from the conformation which appears on the surface of T cells (referred to herein as a "native-like" or "native" conformation). This soluble TCR protein is biologically functional and does not require refolding or renaturation of the protein. The invention also concerns nucleic acid molecules encoding heterodimeric TCR, as well as expression vectors which comprise nucleic acid molecules encoding the heterodimeric TCR, and also host cells containing such expression vectors.

The heterodimeric TCR molecules of the invention can be used to detect and analyze the peptide and MHC/HLA molecular constituents of TCR ligands. The heterodimeric TCR can also be used for diagnostic purposes, such as for the detection of T cells with pathogenic properties. The heterodimeric TCR can additionally be used in functional, cellular and molecular assays, and in structural analyses, including X-ray crystallography, nuclear magnetic resonance spectroscopy, and computational techniques, designed to identify TCR antagonists or agents that inhibit the interaction between TCR and MHC/HLA molecules complexed with antigenic peptides. Similar techniques can be performed to screen for agents capable of blocking the interaction of TCR with TCR specific antibodies. The heterodimeric TCR can additionally be used *in vivo*, in order to compete with pathogenic T cells; or to immunize mammals, particularly humans, against TCR structures that occur on the surface of T cells which perform pathogenic or otherwise undesirable functions. TCR-specific antibodies

raised against heterodimeric TCR can be used in therapeutic strategies that are designed to regulate immune responses *in vivo* by either inhibiting or eliminating specific antigen-recognition by T cells. By selecting antibodies that recognize defined epitopes of the TCR, a restricted subset, or a clone of T cells involved in a disease or medically undesirable immune response, can be targeted. The antibodies can be unmodified, or can also be linked to cytotoxic drugs, toxins, enzymes or radioactive substances.

Brief Description of the Drawings

Figure 1 depicts the coding regions unique to the α-chain (SEQ ID NO. 1) of the soluble, secreted form of the D10 dual chain TCR (dcTCR).

Figure 2 depicts the coding regions unique to the β-chain (SEQ ID NO. 2) of the soluble, secreted form of the D10 dcTCR.

Figure 3 depicts baculovirus transfer vector p9/237 containing the D10 α/β secreted TCR in pAcUW51.

Figure 4A depicts a schematic diagram of variable and constant domains of soluble D10 dcTCR, indicating predicted inter- and intra- chain disulfide bonds.

Figure 4B depicts a schematic diagram of variable and constant domains of the D10-IgG1 TCR construct, indicating predicted inter- and intra-chain disulfide bonds.

Figure 5 depicts baculovirus transfer vector p10/248 containing the B10 α/β secreted TCR in pAcUW51.

Figure 6 depicts baculovirus transfer vector p2/246 containing the B10 TCR α/β TCR-IgG1 chimeras in pAcUW51.

Figure 7 depicts baculovirus transfer vector p7/243 containing the D10 dcTCR α/β TCR-IgG1 chimeras in pAcUW51.

Figure 8 depicts the baculovirus transfer vector p3/598 containing the BDC 2.5 TCR β-IgG1 chimera in pVL941.

Figure 9 depicts the baculovirus transfer vector p7/599 containing the BDC 2.5 TCR α-IgG1 chimera in pVL941.

Figure 10 depicts baculovirus transfer vector p16/599 containing the BDC 2.5 α/β TCR-IgG1 chimeras in pAcUW51 (β/pH, α/p10).

Figure 11 depicts baculovirus transfer vector p20/599 containing the BDC 2.5 α/β TCR-IgG1 chimeras in pAcUW51 (α/pH, β/p10).

Figure 12 depicts nucleic acid (SEQ ID NO. 3) and deduced amino acid (SEQ ID NO. 4) sequences at the 5'-end of the Vα-13.1 gene segment.

Figure 13 depicts baculovirus transfer plasmid p11/606 containing BDC 6.9 TCR β-IgG1 downstream of the polyhedron promoter in pVL941 and BDC 6.9 TCR α-IgG1 downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 14 depicts baculovirus transfer plasmid p33/606 containing BDC 6.9 TCR α-IgG1 downstream of the polyhedron promoter in pVL941 and BDC 6.9 TCR β-IgG1 downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 15 depicts baculovirus transfer plasmid p11/607 containing BDC 2.5 TCR β-IgG1 downstream of the polyhedron promoter in pVL941 and BDC 2.5 TCR α-IgG1-hexahistidine downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 16 depicts baculovirus transfer plasmid p21/607 containing BDC 2.5 TCR β-IgG1 downstream of the polyhedron promoter in pVL941 and BDC 2.5 TCR α-IgG1-streptag downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 17 depicts baculovirus transfer plasmid p41/607 containing BDC 2.5 TCR α-IgG1-hexahistidine downstream of the polyhedron promoter in pVL941 and BDC 2.5 TCR β-IgG1 downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 18 depicts baculovirus transfer plasmid p51/607 containing BDC 2.5 TCR α-IgG1-streptag downstream of the polyhedron promoter in pVL941 and BDC 2.5 TCR β-IgG1 downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 19 depicts baculovirus transfer plasmid p11/608 containing B10 TCR α-IgG1-hexahistidine downstream of the polyhedron promoter in pVL941 and B10 TCR β-IgG1 downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 20 depicts baculovirus transfer plasmid p31/608 containing B10 TCR α-IgG1-streptag downstream of the polyhedron promoter in pVL941 and B10 TCR β-IgG1 downstream of the P10 promoter in pAcUW51. Restriction sites used in the construction are indicated in bold.

Figure 21 depicts baculovirus transfer plasmid p1/258 containing the D10 α-chain downstream of the polyhedron promoter in pVL941.

Figure 22 depicts baculovirus transfer plasmid p7/258 containing the D10 β-chain downstream of the polyhedron promoter in pVL941.

Figure 23 depicts baculovirus transfer plasmid p14/259 containing D10 dcTCR α-hexahistidine downstream of the polyhedron promoter in pVL941.

Figure 24 depicts baculovirus transfer plasmid p9/259 containing D10 dcTCR α-streptag downstream of the polyhedron promoter in pVL941.

Figures 25A and 25B are graphic depictions of the production of antibodies specific for the BDC 2.5 cell-surface TCR by NOD mice immunized with the BDC 2.5 TCR-IgG1 protein. Figure 25(A) is a series of three graphs depicting the following: T cells of the indicated type stained by indirect immunofluorescence with (1) non-immune NOD serum (line) or (2) anti-BDC 2.5 TCR-IgG1 antiserum (long broken line) followed by FITC-GaMIg antibody. TCR

expression was demonstrated by direct immunofluorescent staining with (3) FITC-anti-$V_\beta$-4 mAb (short broken line) for BDC 2.5 and 6.9 cells, and (5) FITC-anti-TCR $C_\beta$ mAb for purified NOD splenic T cells (long-short broken line). Isotype matched controls (4) were FITC-anti-$V_\beta$-6 for the $V_\beta$-4-specific mAb, and FITC-anti-$V_\gamma$-3 for the TCR $C_\beta$-specific mAb (dotted line).

Figure 25(B) is a series of two graphs, depicting the following: BDC 2.5 cells preincubated with (6) buffer alone (line), (7) a 1:10 dilution of pooled NOD mouse non-immune serum (long broken line), or (8) a 1:10 dilution of NOD mouse anti-BDC 2.5 TCR-IgG1 antiserum (short broken line). The cells were then stained with FITC-anti-$V_\beta$-4 mAb, or FITC-anti-TCR $C_\beta$ as indicated.

Figures 26A and 26B are graphic depictions of the blocking of the antigen-specific response of the BDC 2.5 T cell clone *in vitro* by NOD mouse anti-BDC 2.5 TCR-IgG1 antiserum.

Figure 27 is a graphic representation of cytofluorimetry demonstrating Vβ8-specific staining with antiserum from an SJL mouse immunized with D10 TCR-IgG1.

Detailed Description of the Invention

The present invention concerns a polypeptide molecule, or protein, comprising a heterodimeric T cell receptor (TCR). The heterodimeric TCR comprises α and β subunits joined by at least one disulfide bond; the term, "heterodimeric," indicates that the TCR is a disulfide-linked heterodimer comprising separate α and β subunits. The subunits of the heterodimeric TCRs can be chimeric polypeptides ("chimeras") comprising an α or β segment linked to a chimeric partner. The chimeric partner is any polypeptide that allows or promotes heterodimer formation. In a preferred embodiment, the chimeric partner is an

immunoglobulin domain. The chimeric partner can be different for each of the α and β segments, provided that the different partners interact to allow formation of a heterodimer. TCRs comprising chimeric polypeptides are referred to herein as "chimeric" TCR. Alternatively, the subunits of the heterodimeric TCRs can include α and β segments that are not linked to a chimeric partner; TCRs comprising α and β segments without chimeric partners are referred to herein as "dual chain" TCRs (dcTCRs). The term "heterodimeric TCR" encompasses both chimeric TCR and dcTCR.

The heterodimeric TCRs are soluble when purified; moreover, they react with anti-clonotypic antibodies that are specific for the native conformation of the TCR. As described in detail below, soluble heterodimeric TCRs can be produced that are in a conformation that is functionally indistinguishable from the cell surface TCR determinant that is unique to a particular clonal line of T cells.

Several steps are taken to generate heterodimeric TCRs. First, nucleic acid fragments bearing gene sequences for the α and β segments of the TCR of interest are isolated. The nucleic acid fragments can be DNA or cDNA that are isolated by known methods. For example, synthetic oligonucleotide primers corresponding to portions of the α and β gene sequences can be used in the polymerase chain reaction (PCR) to amplify DNA or cDNA prepared from T cells bearing the TCR of interest.

In one embodiment of the invention, soluble heterodimeric TCRs are generated through the use of α-ζ and β-ζ chimeras. To create ζ chimeras, the nucleic acid fragments encoding the α chain and the β chain are each cloned into separate vectors comprising a nucleic acid fragment encoding a ζ chain, thereby generating α-ζ and β-ζ coding regions. The ζ is referred to herein as the "chimeric partner". The α-ζ and β-ζ coding regions are

then inserted into one combination vector with a different promoter for each of the coding regions, or into separate transfer vectors with the same promoter for both coding regions. The combination transfer vector can comprise a single promoter for both the α-ζ and β-ζ coding regions, or separate promoters for each of the coding regions.

In a second embodiment of the invention, soluble heterodimeric TCRs are generated through the use of α-IgG1 and β-IgG1 chimeras. To create IgG1 chimeras, nucleic acid fragments encoding the α chain and the β chain are each cloned into separate vectors comprising a nucleic acid fragment encoding the CH2 and CH3 regions of an IgG1 molecule, thereby generating α-IgG1 and β-IgG1 coding regions. In a preferred embodiment, the vector comprising a nucleic acid fragment encoding a portion of the IgG1 molecule that encodes a nucleic acid fragment encoding the hinge, CH2 and CH3 domains of an IgG1 heavy chain. IgG1 is referred to herein as the "chimeric partner". The α-IgG1 and β-IgG1 coding regions are then inserted into one combination vector with a different promoter for each of the coding regions, or into separate transfer vectors with the same promoter for both coding regions.

In a third embodiment of the invention, soluble dcTCRs are generated through the use of nucleic acid fragments encoding α-ζ and β-ζ chimeras, or α-IgG1 and β-IgG1 chimeras, into which "stop" codons have been inserted near the transmembrane domains of both the α- and β-chains preceding the ζ chain gene, or between the α- and β-chains and the IgG1 coding regions. The nucleic acid sequences encoding the chimeras are generated as described above; "stop" codons are then inserted in the appropriate place. The coding regions are then inserted into a single combination vector with a different promoter for each of the coding regions, or into separate transfer vectors with

the same promoter for both coding regions, as described above.

In a fourth embodiment of the invention, soluble dcTCRs are generated through the use of nucleic acid fragments encoding the $\alpha$ and $\beta$ segments of the TCR of interest, without chimeric partners. The coding regions are then inserted into a single combination vector with a different promoter for each of the coding regions, or into separate transfer vectors with the same promoter for both coding regions, as described above.

The combination vector, or transfer vectors, are expressed in an appropriate vector and host system. A host cell is transformed or transfected with the combination vector or the transfer vectors for replication, transcription and translation. The host cell can be prokaryotic. Gram negative bacterial strains, such as *Escherichia coli*, as well as gram positive bacterial strains, such as *Staphylococcus aureus*, can be used. Alternatively, eukaryotic cells of mammalian or insect origin, or yeast such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* can be used. In a preferred embodiment, the host cell is an insect cell, such as the Sf9 cell line derived from pupal ovarian tissue of *Spodoptera frugipoda*. The combination transfer vectors can be introduced into host cells by various methods known in the art. For example, transfection of host cells with combination transfer vectors can be carried out by electroporation. Other methods can also be employed for introducing fusion protein vectors into host cells; calcium phosphate, calcium chloride or ruthenium chloride mediated transfection, or other techniques, some involving membrane fusion, can be used. In a preferred embodiment, the combination transfer vector is cotransfected with linearized baculovirus DNA into insect cells, such as *Spodoptera frugipoda* (Sf9) cells (Pharmingen, San Diego,

CA) or High 5 *Trichoplusia ni* (SB1-) cells (Invitrogen, San Diego, CA). Such cotransfection results in recombinant baculoviruses encoding the TCR gene constructs (see Luckow, V.A. and M.D. Summers, *Bio/Technology* 6:47-55 (1988)). Appropriate host cells are then infected with the recombinant baculoviruses under conditions that allow amplification of the recombinant viruses and expression of the heterodimeric TCR.

Once a combination transfer vector has been introduced into an appropriate expression system, large amounts of α-ζ and β-ζ subunits, α-IgG1 and β-IgG1 subunits, or α and β segments are expressed. α-ζ and β-ζ subunits interact to form α-ζ/β-ζ complexes on the cell surface, known as "ζ chimeric TCR"; α-IgG1 and β-IgG1 subunits interact to form soluble α-IgG1/β-IgG1 complexes, known as "IgG1-chimeric TCRs"; and α and β segments interact to form soluble α/β complexes, known as "dual chain TCRs" (dcTCRs). Soluble dcTCRs can be generated from the chimeric TCRs by removal of the chimeric partners. For example, soluble dcTCRs are released from α-ζ/β-ζ complexes by digestion with an enzyme that cleaves the subunits near the ζ region. Dual chain TCRs are released from α-IgG1/β-IgG1 complexes in a similar manner.

The heterodimeric TCRs can be purified to homogeneity from host cell lysates by known methods, such as by affinity chromatography and standard biochemical techniques. To facilitate purification, carboxy-terminal extensions can be attached to the α chain region. Representative extensions include hexahistidine or streptag tails. Hexahistidine tails can be used to complex with metal, such as through nickel affinity purification. Streptag tails bind to biotin to take advantage of biotin-streptavidin binding. The heterodimeric TCR can be further purified to maximize yield.

The heterodimeric TCRs can be assayed immunologically using conformation sensitive immunoassays, either before or after proteolytic digestion to liberate free dcTCRs. The heterodimeric TCR can be tested for the presence of the native conformation utilizing the methods described by Engel et al. (*Science 256*:1318 (1992)). These workers transfected the rat basophilic leukemia line RBL-2H3 with recombinant genes encoding the TCR extracellular domains linked to the transmembrane segment and cytoplasmic tail of the $\zeta$ chain. The transfected cells expressed heterodimeric TCR on the cell surface. This TCR could appropriately recognize the stimulatory peptide bound to the I-$E^k$ MHC class II molecule, resulting in MHC-restricted activation of the RBL cells.

Obtaining the unique portion (V$\alpha$V$\beta$) of the TCR in amounts sufficient for biochemical and immunological characterization, and in the correct (native) conformation, is essential for developing a more complete understanding of the immune system. Furthermore, variable regions of TCR may provide drug targets that could potentially be specific for T cells involved in pathological mechanisms. Examples of T cell-mediated pathology in human diseases include: pancreatic $\beta$-cell destruction in insulin-dependent diabetes mellitus (IDDM), demyelination within the central nervous system in multiple sclerosis, pathology in rheumatoid arthritis, and graft rejection following allografting between HLA incompatible individuals. Production of the variable region of the TCR in soluble form is a prerequisite for determining the structure of the TCR involved in disease, and for constructing receptor-ligand assays for screening for TCR antagonists.

The heterodimeric TCR of the invention can be used to derive TCR structures for identification of TCR antagonists or agents that inhibit the interaction between the TCR and

MHC/HLA molecules complexed with antigenic peptides. TCR structures can be applied in rational drug design using computational techniques. TCR structural information derived from one heterodimeric TCR can be used to deduce general rules concerning the whole class of TCR proteins or certain subsets thereof, thereby aiding in the identification of inhibitory compounds. Structural information concerning one particular heterodimeric TCR can be used to devise highly specific inhibitors for a particular T cell clone. Structural information from one heterodimeric TCR can be obtained by standard methods, including information obtained from X-ray diffraction, nuclear magnetic resonance (NMR) spectroscopy, or biochemical or biophysical investigation of the interaction of the heterodimeric TCR with ligands such as MHC/HLA molecules complexed with antigenic peptide or superantigen, or TCR-specific antibodies. Superantigens are proteins that share the ability to bind to human and mouse HLA/MHC Class II proteins to form a ligand complex for the V$\beta$ segment of the TCR. Because it binds to V$\beta$ segments belonging to particular families, a superantigen-HLA/MHC Class II complex can stimulate many more T cells than a complex of a particular Class II molecule and an antigenic peptide. Superantigens are represented by the Staphylococcal enterotoxins and Streptococcal toxins (Marrack, P. and Kappler, J., *Science 248*:705-711, 1990), and by proteins encoded by endogenous retroviruses (for example, Woodland, D.L. *et al.*, *Nature 349*:529-530 (1991)). Once structural information concerning one TCR is obtained, it can then be used to solve the crystallographic structure of other TCR by molecular replacement techniques. Consequently, structural coordinates of any TCR can be used in the determination of the structure of TCR of pathological importance in mammals, particularly humans.

The heterodimeric TCR of the invention can additionally be utilized in assays to screen for agents that inhibit the interaction of TCR with: 1) complexes formed between MHC/HLA molecules and antigenic peptides or superantigens (referred to herein collectively as antigens), and 2) TCR specific antibodies, including but not limited to anti-clonotypic antibodies. Such agents include TCR blockers or antagonists, MHC/HLA blockers or antagonists, and molecular mimics of the TCR ligands. To conduct the assay for agents that inhibit the interaction of TCR with the complexes formed between MHC/HLA molecules and antigenic peptides or antigens, a sample of isolated and purified heterodimeric TCR is incubated with the MHC/HLA molecules and antigenic peptides or superantigens of interest, under conditions that allow the heterodimeric TCR to interact with the MHC/HLA molecules and antigenic peptides/superantigens. This sample is the control sample. A second sample (the test sample) identical to the control sample except that it is exposed to the agent to be tested, is also incubated under the same conditions. Both the control sample and the test sample are then evaluated to determine the level of interaction of TCR with the complexes formed between the MHC/HLA molecules and antigenic peptides or superantigens of interest. If less interaction occurs in the presence of the agent to be tested (in the test sample) than in the absence of the agent to be tested (in the control sample), then the agent is an inhibitor of the interaction between TCR and the complexes formed between the MHC/HLA molecules and antigenic peptides or superantigens of interest. To conduct the assay for agents that inhibit the interaction of TCR with TCR specific antibodies, an assay similar to that described above is conducted, using a sample of isolated and purified heterodimeric TCR that is incubated with the TCR specific antibody of interest as the control

sample. Less interaction between the heterodimeric TCR and the antibody in the presence of the agent to be tested, than in the absence of the agent to be tested is indicative that the agent is an inhibitor of the interaction between TCR and the TCR specific antibody of interest.

The heterodimeric TCR of the invention can also be used to detect the MHC/HLA molecular constituents of TCR ligands using molecular assays. Recombinant, soluble forms of MHC/HLA molecules can be immobilized on a solid support. Synthetic and/or naturally occurring peptides can be incubated with the MHC/HLA molecules to form complexes that can be investigated for their ability to bind heterodimeric TCR added in the solvent phase. Binding of the receptor proteins can be detected utilizing TCR-specific antibodies and standard ELISA, or by surface plasmon resonance using the BIAcore™ (Pharmacia, Piscataway, NJ) biosensor system (Fagerstam, L., *Tech. Prot. Chem. 2*:65-71 (1991); Malmqvist, M., *Current Biology 5*:282-286 (1993)). Identification of ligands recognized by T cells that are involved in disease states, such as those involved in the destruction of pancreatic $\beta$-cells in insulin-dependent diabetes mellitus (IDDM), would allow the establishment of cellular or molecular screening assays for agents that block activation of pathogenic T cells by interference with the binding of the T cell receptor to its ligand. Such assays would be conducted in a similar manner to the assays described above: a sample of isolated and purified heterodimeric TCR of interest (i.e., heterodimeric TCR that has a native-like conformation that is functionally indistinguishable from that present on the pathogenic T cells, generated by the methods described above) and its ligand is incubated under conditions that allow interaction between the heterodimeric TCR and its ligand; a second sample of heterodimeric TCR and ligand is exposed to the agent to be tested and incubated in a similar manner. The

level of interaction between the heterodimeric TCR and ligand is then examined; a lower level of interaction in the presence of the agent than in the absence of the agent is indicative of the ability of the agent to block activation of the heterodimeric TCR, and thus to block activation of the pathogenic T cells. Agents that could block activation of pathogenic T cells include antibodies to T cell receptors, such as those described below.

The heterodimeric TCR of the invention can also be used to generate antibodies, either monoclonal or polyclonal, using standard techniques. The term "antibody", as used herein, encompasses both polyclonal and monoclonal antibodies, as well as mixtures of more than one antibody reactive with heterodimeric TCR (e.g., a cocktail of different types of monoclonal antibodies reactive with heterodimeric TCR). The term antibody is further intended to encompass whole antibodies and/or biologically functional fragments thereof, chimeric antibodies comprising portions from more than one species, humanized antibodies and bifunctional antibodies. Biologically functional antibody fragments which can be used are those fragments sufficient for binding of the antibody fragment to heterodimeric TCR. Once the antibodies are raised, they are assessed for the ability to bind to heterodimeric TCR. Conventional methods can be used to perform this assessment.

The chimeric antibodies can be derived from two different species (e.g., a constant region from one species and variable or binding regions from another species). The portions derived from two different species can be joined together chemically by conventional techniques or can be prepared as single contiguous proteins using genetic engineering techniques. DNA encoding the proteins of both the light chain and heavy chain portions of the chimeric antibody can be expressed as contiguous proteins.

Monoclonal antibodies (mAb) reactive with heterodimeric TCR can be produced using somatic cell hybridization techniques (Kohler and Milstein, *Nature 256*: 495-497 (1975)) or other techniques. In a typical hybridization procedure, a crude or purified heterodimeric TCR protein, or peptide derived from the heterodimeric TCR protein, can be used as the immunogen. An animal is immunized with the immunogen to obtain anti-heterodimeric TCR antibody-producing spleen cells. The species of animal immunized will vary depending on the specificity of mAb desired. The antibody producing cell is fused with an immortalizing cell (e.g., a myeloma cell) to create a hybridoma capable of secreting anti-heterodimeric TCR antibodies. The unfused residual antibody-producing cells and immortalizing cells are eliminated. Hybridomas producing desired antibodies are selected using conventional techniques and the selected hybridomas are cloned and cultured.

Polyclonal antibodies can be prepared by immunizing an animal in a similar fashion as described above for the production of monoclonal antibodies. The animal is maintained under conditions whereby antibodies reactive with heterodimeric TCR are produced. Blood is collected from the animal upon reaching a desired titer of antibodies. The serum containing the polyclonal antibodies (antisera) is separated from the other blood components. The polyclonal antibody-containing serum can optionally be further separated into fractions of particular types of antibodies (e.g., IgG, IgM).

The antibodies of the invention can be used to detect T cells with pathogenic properties in mammals, including humans. To detect pathogenic T cells, a sample of lymphocytes is incubated with antibodies to the heterodimeric TCR of interest (the heterodimeric TCR that has a native-like conformation that is functionally

indistinguishable from that present on the pathogenic T cells, generated by the methods described above). Interaction between the lymphocytes and the antibodies is assessed; the presence of interaction between the lymphocytes and the antibodies is indicative of the presence of pathogenic T cells. The lymphocytes can be obtained, using standard techniques, from peripheral blood, bodily fluids (including cerebrospinal fluid and synovial fluid), and lymph nodes, or spleen or other tissue biopsy specimens. Analysis of the lymphocytes can be performed before or after *in vitro* culture of the lymphocytes.

The antibodies of the invention can also be used to deplete T cells or inhibit T cell activation *in vivo* in mammals, including humans. Therapeutic regimens can be designed in which antibodies are administered, using standard methods, in order to inhibit antigen recognition, by binding to T cell surface TCR and thereby sterically blocking the interaction between the variable region of the TCR and the specific complex of antigenic peptide and MHC molecule. Alternatively, or in addition, the complexes formed between the TCR-specific antibodies and the cell surface TCR can deplete T cells by utilizing accessory elements of the immune system that destroy the antibody-bound T cell. It is anticipated that the Fc region of antibodies bound to TCR on the T cell surface will engage and activate cytotoxic mechanisms mediated by the complement system, macrophages, monocytes, or antibody-dependent cytotoxic cells. The efficiency of T cell depletion can be enhanced by administering TCR-specific antibodies that are covalently conjugated to a cytotoxic or anti-metabolic agent, such as toxins of microbial or synthetic origin, including peptide toxins or polypeptides related to toxins (Frankel, A.E., *J. Biol. Response Mod.* *4*:437-446 (1985)); enzymes; radioactive substances; or cytotoxic drugs (Hawkins, R.E., *et al.*, *British Medical*

*Journal 305*:1348-1352 (1992)). In applications of TCR-specific antibodies *in vivo* as immune response modifiers, immunoregulators or immunosuppressors, the selection of antibodies with defined specificity allows targeting of either the whole T cell population, or a defined T cell sub-population, within an individual animal or human. For example, antibodies specific for a clonotypic epitope would target only the members of a single T cell clonotype, whereas antibodies specific for a Vβ family-specific epitope would target all the T cell clones bearing TCR utilizing Vβ-segments belonging to that particular family. Only those T cells involved in a particular disease or medically undesirable immune response would be targeted for modulation or elimination; the majority of T cells involved in the maintenance of immunity against infectious agents would be spared. Antibodies can be administered directly; alternatively, they can be administered indirectly, such as by maternal transmission (transplacental transmission to offspring of a mammal during gestation, or by transmission during nursing). The pregnant or nursing female is immunized with the TCR of interest, and maternally generated antibodies are passively transmitted to the offspring before and/or during birth, and/or after birth during nursing. The antibodies to the TCR are administered to a mammal in a therapeutically effective amount, which is the amount of the antibody that is necessary to inhibit the activation of, deplete or eliminate the pathogenic T cells. In indirect administration of antibodies an amount of TCR is administered to the mother of the offspring that is sufficient to generate an antibody response in the mother.

The heterodimeric TCR of the invention can also be used *in vivo* in mammals, including humans, to compete with pathogenic T cells for their specific MHC/HLA class II associated peptide antigen. In this manner, the heterodimeric TCR can be used to deplete antigen such that

the activation of the pathogenic T cells would be reduced or eliminated in vivo. Pathogenic T cells of interest include those which are involved in pancreatic β-cell destruction in insulin-dependent diabetes mellitus (IDDM), demyelination within the central nervous system in multiple sclerosis, pathology in rheumatoid arthritis, and graft rejection following allografting between HLA incompatible individuals. The heterodimeric TCR are administered to a mammal in a therapeutically effective amount, which is the amount of the heterodimeric TCR that is necessary to inhibit the activation of, deplete or eliminate the targeted T cells.

The heterodimeric TCR of the invention can also be used to immunize mammals, including humans, against TCR antigenic structures that occur on the surface of T cells which perform pathogenic or otherwise undesirable functions (the "targeted T cells"), such as graft rejection following transplantation. Such T cells can be identified in samples of peripheral blood, or in biopsy specimens taken from lymphoid organs or sites of inflammation. Lymphocytes in a sample are purified and investigated *in vitro* for their ability to make a T cell dependent proliferative response to the relevant MHC/HLA associated antigenic epitope. The T cells that undergo cell division can be established *in vitro* as lines or clones from which TCR genes can be cloned and used to produce heterodimeric TCR by the recombinant DNA technology described herein. TCR antigenic structures include clonotypic epitopes, Vα or Vβ family-specific epitopes, conformational epitopes, and linear epitopes. Immunization against TCR antigenic structures that occur on the surface of the targeted T cells inhibits the activity of the targeted T cells, thereby reducing the pathogenic or undesirable effects of the targeted T cells. To immunize a mammal, the heterodimeric TCR are administered to a mammal in an effective amount, which is the amount of the

heterodimeric TCR that is necessary to inhibit the activity of the targeted T cells.

Administration of heterodimeric TCR, whether it be for the reduction or elimination of the activation of pathogenic T cells or for immunization, can be in the form of a single dose, or a series of doses separated by intervals of days or weeks. The term "single dose," as used herein, can be a solitary dose, and can also be a sustained release dose. The heterodimeric TCR can be administered subcutaneously, intravenously, intramuscularly, intraperitoneally, orally, by nasal spray or by inhalation, opthamologically, topically, via a slow-release compound, or via a reservoir in dosage formulations containing conventional, physiologically-acceptable carriers and vehicles. Alternatively, a DNA fragment encoding the heterodimeric TCR can be administered. The formulation in which the heterodimeric TCR is administered will depend in part on the route by which it is administered, and the desired effect.

The following examples are further illustrative of the present invention. These examples are not intended to limit the scope of the present invention.

## EXAMPLE 1 Production of Heterodimeric T Cell Receptors as ζ Chimeras

### A. Materials

D10 clonotype-specific monoclonal antibody (mAb) 3D3 was provided by A. Bothwell (Yale Medical School, New Haven, CT). H28-710-16 (H28) and H57-597 (H57) are Cα- and Cβ-specific monoclonal antibodies, respectively (Kubo, R. T. *et al.*, *J. Immunol.* *142*:2736-2742 (1989); Becker, M.L. *et al.*, *Cell* *58*:911-921 (1989)); they were obtained from E. Reinherz (Dana Farber Cancer Institute, Boston, MA). Vα2-

specific mAb B20.1.1 (Pircher, H. *et al.*, *Eur. J. Immunol.* *22*:399-404 (1992)) was provided by P. Fink (U. Washington, Seattle, WA). Biotinylation of mAbs H28 and H57 was carried out as described (Harlow, E. and D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)). The following family-specific mAbs were purchased from Pharmingen, Inc. (San Diego, CA): anti-Vα11 mAb (Jameson, S.C. *et al.*, *J. Immunol.* *146*:2010 (1991)); anti-Vβ3 (Vβ3; Sugihara et al. *J. Immunol.* *150*:683 (1993)); and anti-Vβ8.1/8.2 (Vβ8; Kanagawa, O., *J. Exp. Med.* *170*:1513 (1989)).

Anti TCR V-region, family specific monoclonal antibodies were purchased from Pharmingen, Inc., (San Diego, CA) apart from the $V_\beta 4$ family specific antibody which was purchased from Harlan Bioproducts for Science (Indianapolis, IN).

## B. Construction of D10 α and β Chains as ζ Chimeras in Baculovirus Vectors

### 1. Construction of Plasmids Encoding D10 dcTCR as α-ζ and β-ζ Chimeras

The plasmid ph3C3ζ, containing a human CD3 ζ-chain cDNA, was obtained from the laboratory of E. Reinherz (Dana Farber Cancer Institute, Boston, MA). A BamHI-EcoRI restriction fragment spanning the transmembrane and cytoplasmic coding regions of the CD3 ζ-chain was excised from the plasmid and cloned between the BamHI and EcoRI restriction sites within the polylinker of the vector pAcC5 (Luckow, V.A. and M.D. Summers, *Bio/Technology* *6*:47-55 (1988)). This created p2/220, a plasmid which contains the appropriate region of the ζ-chain preceded by NcoI and BamHI restriction sites, and followed by EcoRI and BglII restriction sites.

DNA encoding the D10 α- and β- chains was amplified, using the polymerase chain reaction (PCR) from the vectors pFRSV-SRαD10α GPI-TCR and pFRSV-SRβD10Bβ GPI-TCR obtained from Dr. A. Bothwell (Yale Medical School, New Haven, CT). The PCR reactions were done using oligonucleotides that were designed to amplify the extracellular V, J and C regions of the α chain, and V, D, J and C regions of the β chain, from the methionine initiation codons of the signal sequences to the codons for the first amino acid after the conserved cysteines involved in the inter-chain sulfhydryl bond of the α/β TCR. The primers used to amplify the α-chain were 5′-CCTCTAGAAGATCTCCATGGACAAGATCCTGACAGCATCATGTTTACTC-3′ (SEQ ID NO. 5) and 5′-GGAATTCAGATCTGATGGAGCAGTCGTTGATCCACGTGGTACCAGGTCTGCTGATGAACAGGGGACGTCTGAACTGGGGTAGGTGGC-3′ (SEQ ID NO. 6). For the β-chain, the primers used were 5′-CCTCTAGAAGATCTCATGAGTAACACTGCCTTCCCTGACCCCGCC-3′ (SEQ ID NO. 7) and 5′-GGAATTCAGATCTCCGTCTAGTCGTGATGAACCACGAGGTACCAGATCAGCAGACGAACAGTCTGCTCGGCCCCAGGCCTCGGCCGAGATGTTCTGTGTGACAG-3′ (SEQ ID NO. 8). At the 3′-end of the TCR constant regions these primers introduced sequence encoding a short linker region and a thrombin cleavage site. Silent mutations were also incorporated to provide convenient restriction sites. PCR was performed using Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) under conditions recommended by the supplier for 30 cycles at 95°C/45", 55°C/45", 72°C/90".

The PCR products were restricted and cloned between the XbaI and EcoRI sites in the polylinker of pSP72 (Promega, Madison, WI). The sequences of the TCR genes were determined using Sequenase (U.S.B., Cleveland, OH) according to the manufacturer's recommendations. They were found to encode the wild type amino acid sequences (Figures 1 and 2) except for a mutation creating a glutamine codon

in the β-chain C-region. The mutation was discovered in the cloned cDNA, and was not due to infidelity of the Taq polymerase. A fragment with the correct codons for the erroneous region was substituted to revert the mutation so as to encode the wild-type arginine. The sequences of the cloned genes were verified by sequence analysis. The recombinant plasmids were named p1/226 (α) and p6/227 (β).

In order to join the TCR and ζ-chain genes in frame, the D10 α gene, excised from p1/226 as a NcoI-BglII fragment, and the D10 β-gene, excised from p6/227 as a BspHI-BglII fragment, were individually cloned between the NcoI and BamHI sites of p2/220, to create the plasmids p3/228 (α-ζ) and p2/229 (β-ζ).

Two fragments, a BglII-HindIII fragment from p1/226, and a HindIII-BglII fragment from p3/228, which together encode the α-ζ chimera, were cloned into the BamHI site of pVL941 (Pharmingen, San Diego, CA) to create p2/230. Similarly, a BglII-NcoI fragment from p6/227 and a NcoI-BglII fragment from p2/229, together encoding the β-ζ chimera, were cloned into the BamHI site of pVL941 to create p1/231. These two plasmids encode either the α-ζ or β-ζ chimera under the control of the polyhedron promoter. The above-mentioned α-ζ- and β-ζ- encoding fragments were sequentially cloned into pAcUW51 (Pharmingen, San Diego, CA) at the BamHI and BglII sites, respectively, to create p11/231. p11/231 encodes the α-ζ chimera under control of the polyhedron promoter, and the β-ζ chimera under control of the P10 promoter.

2. Transfection into Sf9 cells

The vectors described above were used with BaculoGold (Pharmingen, San Diego, CA) linearized baculovirus DNA to cotransfect insect cells in order to obtain a recombinant Baculovirus encoding a TCR gene. The BaculoGold system yields only recombinant viruses that contain the gene of

interest due to a lethal deletion in the BaculoGold DNA that permits viable virus production only after homologous recombination with a baculovirus transfer vector that contains the appropriate region to complement the lesion in BaculoGold DNA.

Sf9 cells, a subclone of the IPLB-Sf21-AE line, or High 5 cells, were propagated at 27°C, either in suspension or as monolayer cultures in either TMN-FH media (JRH Biosciences, Lawrence, KS) supplemented with 10% heat-inactivated FCS, 10 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma, St. Louis, MO) or in serum-free media, SF900II (Gibco, Life Technologies, Bethesda, MD) or Excell 400 (JRH Biosciences, Lawrence, KS), supplemented with antibiotics.

Transfer plasmid and linearized BaculoGold viral DNA were co-transfected into Sf9 cells according to the manufacturer's recommendations (Pharmingen, San Diego, CA). Briefly, 2 x $10^6$ Sf9 cells were seeded onto a 60 mm tissue culture plate and the cells were allowed to attach for 1 hour at 27°C. 0.5 μg of linearized BaculoGold viral DNA was mixed with 2-3 μg of transfer plasmid DNA, including the gene of interest, in a sterile microfuge tube. After five minutes at room temperature, 1 ml of transfection buffer B (25 mM HEPES pH 7.1, 140 mM NaCl, 125 mM $CaCl_2$) was added to the DNA. The media was aspirated from the attached cells and 1 ml of transfection buffer A (Grace's medium containing 10% FCS) was added. One ml of the transfection buffer B/DNA solution was added drop-wise to the plate while it was gently rocked. The plate was then incubated at 27°C for 4 hours. After the 4 hours, the medium was removed from the plate and 5 ml of TMN-FH medium with 10% fetal calf serum (FCS) was added. After the incubation at 27°C for 6 days, the supernatant containing the recombinant viruses was harvested and titrated to

determine the concentration of plaque forming units (pfus). Clonal isolates of virus were generated by one or more rounds of plaque purification as previously described (Summers, M.D. and G.E. Smith, *Tex. Agric. Exp. St. Bull. No. 1555*, 10-39 (1988); O'Reilly, D.R. *et al.*, *Baculovirus Expression Vector: A Laboratory Manual*, W.H. Freeman and Co., N.Y., (1992)). The clonal isolates were tested for expression of D10 dcTCR upon infection of host cells.

C. Titration of Virus by Plaque Assay

Both purified and unpurified viruses were titrated by plaque assay as previously described (Summers, M.D. and G.E. Smith, *Tex. Agric. Exp. St. Bull. No. 1555*, 10-39 (1988); O'Reilly, D.R. *et al.*, *Baculovirus Expression Vector: A Laboratory Manual*, W.H. Freeman and Co., N.Y., (1992)). Briefly, 60 mm tissue culture plates were seeded with 2 x $10^6$ Sf9 cells; the cells were allowed to attach for 1 hour at 27°C. The virus stock was diluted in TMN-FH medium with 10% FCS in dilutions of $10^{-4}$, $10^{-5}$, $10^{-6}$, and $10^{-7}$. The culture medium was aspirated from the cells and 0.5 ml of each virus dilution was added to triplicate sets of plates. The infection was rocked gently at room temperature for 1 hour to allow the virus particles to attach to the cells. The supernatant was then aspirated from the cells and 4 ml of 0.5% agarose, SEAKEM ME grade (FMC Bio-Products, Rockland, ME) in TMN-FH medium with 10% FCS was added as an overlay to each plate. The agarose was allowed to harden and the plates were then incubated at 27°C for 4 days, after which the plates were stained with 100 μg/ml neutral red (Sigma, St. Louis, MO) in 0.5% agarose in PBS. The plates were incubated overnight at 27°C and the plaques, which appeared as clear circular areas against a dark red background, were then counted to

determine the titer of the virus preparation (pfu/ml). Neutral red stains live cells.

D. Amplification of Virus for a High Titer Stock

Sf9 cells were seeded at 3 x $10^5$ cells/ml in 800 ml of TMN-FH media with 10% fetal calf serum and grown in suspension in a 1 liter spinner flask. In an initial experiment the cells were counted at various times to determine when the cells were in log phase. In the virus amplification step, the cells were infected when in early to mid log phase (8 x $10^5$ cells/ml) with the recombinant virus of interest at a multiplicity of infection (MOI) of 0.1 plaque forming virus particles per cell. The infection was allowed to incubate at 27°C for 5 days post-infection (pi). The resulting high titer virus supernatant was then harvested and cleared of cells and debris by centrifugation for 20 minutes at 1500 rpm and filtered through 0.22 micron filter unit. The supernatant was stored at 4°C and titered as described above to determine the number of pfus/ml. Virus was amplified no more than two times before use.

To produce protein for purification, 6- to 8-L cultures of Sf9 or High 5 cells in serum-free media were grown (Rice, J.W. *et al.*, *Biotechniques* 15:1052-1059 (1993)) in 8-L spinner flasks equipped with overhead drive systems (Bellco Glass, Vineland, NJ) to a density of 0.8 to 1.2 x $10^6$ cells/ml and infected at an MOI of 5. Media were harvested three days post infection by centrifugation for 30 minutes at 4.5K rpm and filtration through a 0.2-micron filter.

E. Analysis of Surface Expression of TCR

Expression was tested on a small scale. Each 100 mm tissue culture plate was seeded with 5 x $10^6$ Sf9 cells in

TMN-FH medium with 10% FCS, and infected with recombinant baculovirus at an MOI of 10. Three days post infection, cells or media were harvested and assayed by FACS (Becton Dickinson, San Jose, CA) or ELISA (see below, Example 4) after treatment with thrombin.

1. Treatment with Thrombin to Release Soluble TCR

Sf9 cells were infected with the recombinant D10 $\alpha$-$\zeta$, $\beta$-$\zeta$ baculovirus (from transfer vector p11/231) as described above. Three days after infection, the cells were harvested from the plate and spun at 1000 rpm for 8 min. The cell pellet was resuspended in a buffer containing 150 mM NaCl, 3.5 mM $CaCl_2$, and 50 mM Tris-HCl (pH 8.0). The cells were divided into several tubes at ~1 x $10^6$ cells per tube. The resuspended cells were incubated at room temperature for one hour in the presence of from 0 - 20 units of thrombin (Calbiochem, San Diego, CA). To prepare the enzyme, 500 units of thrombin were resuspended in 300 $\mu$l 50 mM Tris-HCl (pH 8.0), 2 mM $CaCl_2$. After digestion, the cells were spun down at 1000 rpm for 5 minutes, rinsed in PBS with 2% Fetal calf serum (FCS) and stained with FITC/mAbs recognizing the cell surface TCR (either mAb 3D3, or V$\alpha$2- or V$\beta$8-specific mAbs).

2. FACScan Analysis

Analysis of the samples by cell-surface immunofluorescence indicated that 5-30% of the cell surface TCR was removed by the thrombin treatment.

FACS analyses were performed on Sf9 insect cells that were infected with various recombinant viruses, as described above. The V$\alpha$2- and V$\beta$8-specific mAbs react with the V-regions of the D10 dcTCR. V$\alpha$11 and V$\beta$3 family-specific mAbs were used as controls. Bright staining of Sf9 insect cells infected with recombinant virus v11/231

(derived from transfer plasmid p11/231 encoding both the α-ζ and β-ζ chimeras) was observed with mAb 3D3, the D10 clonotype-specific mAb. Identical results were obtained when the separate α-ζ- and β-ζ- encoding viruses (derived from transfer plasmids p2/230 and p1/231, respectively) were used to co-infect Sf9 insect cells. When the α-ζ or β-ζ encoding plasmids were used independently, no 3D3 mAb reactive epitope appeared on the surface of these cells, although the Vα2 and Vβ8 epitopes were expressed. The results are summarized in Table I.

Table I. FACS Analyses

| Infecting Virus | mAb Reactivity | | | | |
|---|---|---|---|---|---|
| | 3D3 | Vα2 | Vβ8 | Vα11 | Vβ3 |
| 11/231 α-ζ, β-ζ | + | + | + | - | - |
| α-ζ | - | + | - | - | - |
| β-ζ | - | - | + | - | - |
| Mock Infection | - | - | - | - | - |

These results indicate that the D10 dcTCR ζ-chain chimera assembles as an α/β heterodimer on the surface of infected insect Sf9 cells. Thrombin cleavage of such infected cells released soluble TCR that was reactive in both the ELISA formats described below in Example 4.

EXAMPLE 2 Synthesis of Chimeras Containing Stop Codons to Generate dcTCR

In order to avoid the requirement for thrombin digestion to release soluble TCR, a different recombinant virus, v9/237, was generated.

A. Construction of Plasmids Encoding D10 dcTCR α and β Extracellular Domains

To construct a transfer plasmid encoding a secretable form of the D10 dcTCR, stop codons were introduced into p11/231 (described above) at the ends of both TCR α- and β-genes immediately following the codons encoding the first amino acid after the conserved inter-chain disulfide-forming cysteine residues and before the beginning of the ζ-chain genes. Two synthetic oligonucleotides, designed to introduce two stop codons after the TCR α-chain gene, 5′-CCCCTGTGATTAATGAGGTAC-3′ (SEQ ID NO. 9) and 5′-CTCATTAATCACAGGGGACGT-3′ (SEQ ID NO. 10), were annealed to create a DNA duplex having a cohesive, AatII-compatible, 5′-end and a cohesive, KpnI-compatible, 3′-end. This molecule was cloned between the AatII and KpnI sites introduced by the PCR primers at the 3′-end of the D10 α-gene. Similarly, two oligonucleotides, 5′-GGCCGAGGCCTGGGGCCGAGCAGACTGTGGGTGATAACCATGGTAC-3′ (SEQ ID NO. 11) and 5′-CATGGTTATCACCCACAGTCTGCTCGGCCCCAGGCCTC′-3′ (SEQ ID NO. 12), were annealed and cloned between the EagI and KpnI sites, also introduced by PCR, at the 3′-end of the D10 β-gene. This created the transfer plasmid, p9/237 (shown in Figure 3), encoding the truncated D10 α- and β-chains with no transmembrane or cytoplasmic domains.

The transfer plasmid p1/258 (Figure 21), encoding only the D10 α-chain, was constructed by cloning into the BamHI site of pVL941 (Pharmingen, San Diego, CA), two α-chain-encoding fragments, a BglII-HindIII fragment excised from 1/226, and a HindIII-BglII fragment from pBDC 6.9 α(f4). The transfer plasmid p7/258 (Figure 22), encoding only the D10 β-chain, was similarly constructed by using two β-chain-encoding fragments, a BglII-Bpu1102I fragment from p9/237 and a Bpu1102I-BglII fragment from pCR-BDC 6.9 β(f4). pCR-BDC 6.9 α(f4) and pCR-BDC 6.9 β(f4) contain BDC 6.9 TCR α- and β-chain genes truncated prior to the

transmembrane regions at the same locations described here for the D10 dcTCR. BglII sites occur immediately 3' of the stop codons.

The nucleotide sequences encoding the soluble, extracellular domains of the D10 dcTCR are shown in Figures 1 and 2. A schematic representation of the truncated D10 dcTCR is depicted in Figure 4A.

B. Synthesis of Vectors to Express Soluble B10 TCR

To construct a vector, p10/248 (Figure 5), encoding a secretable form of the B10 TCR, D10 Vα- and Vβ-encoding sequences within the plasmid p9/237 were sequentially replaced with appropriate restriction fragments encoding the Vα- and Vβ-regions of the B10 TCR. First, a plasmid p1/244, was constructed by replacing the D10 Vα-encoding sequence within the plasmid p3/236, between the BamHI site immediately downstream of the polyhedrin promoter and the NcoI site within the constant region, with a B10 Vα-encoding BamHI-NcoI restriction fragment from the plasmid pB10α2 (Fink, P.J. *et al.*, *Nature* *321*:219-223 (1986)). p3/236 was an intermediate in the construction of p9/237 and contains the D10 α-gene followed by stop codons and the untranslated ζ-chain gene. Next, p10/248 was constructed by cloning both a B10 Vβ-encoding XbaI-EagI fragment from p2/246 (described below; see Figure 6) and a EagI-BglII fragment from p9/237 containing the stop codons after the β-gene between the XbaI and BglII sites of p1/244. p10/248 contains the B10 α-gene downstream of the polyhedrin promoter and the B10 β-gene downstream of the P10 promoter, both followed by stop codons.

EXAMPLE 3 Production of Heterodimeric T Cell Receptors as IgG1 Chimeras

A. Construction of Plasmids Encoding the D10 dcTCR Genes as IgG1 Chimeras

cDNA encoding the hinge, CH2 and CH3 domains of the IgG1 heavy chain was amplified from total RNA isolated from the IgG1 secreting hybridoma 3D3. Total RNA was prepared from approximately 1 x $10^7$ 3D3 cells using RNazol B (Tel-Test, Friendswood, TX) according to the manufacturer's recommendations. One μg of RNA was incubated with Superscript reverse transcriptase (Gibco/BRL, Bethesda, MD) according to the manufacturer's instructions using 0.1 μg of oligo (dT) as primer. Five percent of this reaction mixture provided a template for PCR (performed as described above) using the oligonucleotide primers 5′-GCTGGTACCCAGGGGTAGTGGTTGTAAGCCTTGCATATGTACAGTCCCAGAAGTA-3′ (SEQ ID NO. 13) and 5′-GAAGATCTCATTTACCAGGAGAGTGGGAGAGGCTCTTCTC-3′ (SEQ ID NO. 14) to yield a single product of the expected size. Triplicate reactions were pooled, purified, and cloned into the vector pCRII (Invitrogen, San Diego, CA). A plasmid pIgG1(1a)/584, containing the coding sequence of the hinge, and CH2 and CH3 domains of the IgG1 heavy chain, was thus obtained. The IgG1-encoding sequence contained in this plasmid was determined to be identical to the published sequence (French, D.L. *et al.*, *J. Immunol.* *146*:2010 (1991)).

Both ζ-chain genes within p11/231, described above, were sequentially replaced with the IgG1 sequence excised from pIgG1(1a)/584 as a KpnI-BglII fragment. This created p7/243 containing the D10 α-IgG1 gene under control of the polyhedrin promoter, and the D10 β-IgG1 gene under control

of the P10 promoter. The schematic representation of D10-IgG1 TCR chimera is shown in Figure 4B.

B. Synthesis of Vectors Encoding the B10 TCR Genes as IgG1 Chimeras

Construction of pAcUW51 with D10 α and β as IgG1 chimeras, p7/243 (Figure 7), was carried out to increase the immunogenicity of the TCR for immunoregulatory studies. The PCR product of IgG1 from pIGG1(1a)/584 was digested with KpnI & BglII and used to replace an equivalently bounded ζ chain gene behind both the D10 α- and D10 β-genes in the pAcUW51 derived, double promoter transfer vector(p11/231).

A vector encoding the B10 TCR as IgG1 chimera was also constructed. First, a plasmid, p7/245, containing the B10 TCR α-IgG1 chimeric gene downstream of the polyhedrin promoter was constructed. This was accomplished by replacing the Vα-encoding region of the D10 TCR gene between the BstYI site (generated by the ligation of BamHI- and BglII-digested restriction fragments) immediately downstream of the promoter and the NcoI site within the Cα-region, contained in the plasmid p1/242, with a Vα-encoding BamHI-NcoI gene fragment from the plasmid pB10α2 described above. p1/242 was an intermediate in the construction of p7/243 and contains the D10 TCR α-IgG1 gene downstream of the polyhedrin promoter. In order to create the plasmid p2/246 containing the B10 TCR α-IgG1 downstream of the polyhedrin promoter and the B10 TCR β-IgG1 downstream of the P10 promoter, the B10 TCR β gene was first removed from the plasmid pB10β1 (Fink, P.J. et *al.*, *Nature* *321*:219-223 (1986), provided by S. Hedrick, University of California, San Diego, CA), and cloned into the vector pFRSV-SRβD10β GPI-TCR (described above) so as to replace the D10 Vβ-encoding region with the Vβ-encoding region of the B10 TCR. This generated the plasmid p1/245, containing the B10 β-

gene with an upstream BamHI site. Next, the B10 TCR β-IgG1 gene was created and cloned into the plasmid p7/245 to generate p2/246. This was accomplished by cloning two fragments, a BamHI-NcoI fragment from p1/245, and a NcoI-BglII fragment from p7/243, into the BglII site of p7/245 immediately downstream of the P10 promoter.

C. Synthesis of Vectors Encoding the BDC 2.5 TCR Genes as BDC 2.5 TCR-IgG1 Chimeras

The TCR α and β coding sequences from the T-cell clone BDC 2.5 were amplified from RNA as follows. Total RNA was prepared from approximately 5 million BDC 2.5 cells (K. Haskins, Barbara Davis Center for Childhood Diabetes, Denver, CO) by an RNazol B (Tel-Test, Friendswood, TX) procedure (Chomczynski *et al.*, *Anal. Biochem. 162*:156 (1987)). One μg of RNA was reverse transcribed with 20 ng of oligo (dT) at 42°C in a 20 μL volume using Superscript reverse transcriptase (BRL, Bethesda, MD) according to the manufacturers recommendations. The TCR α and β genes were amplified from 1 μL of this cDNA preparation under the following conditions: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 1.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 units of AmpliTaq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT), and 270 nM each of either the Vβ4 and 3′ D10 β-oligonucleotide (5′-CCTCTAGAAGATCTCCATGGGCTCCATTTTCCTCAGTT-3′; SEQ ID NO. 15) or the Vα1 and 3′ D10 α-oligonucleotide (5′-CCTCTAGAAGATCTGCATGCATTCCTTACATGTTTCACTA-3′′ SEQ ID NO. 16). Seventy-five μL PCRs were setup using Perkin-Elmer PCR Gems according to the manufacturers suggestions. These were cycled 30 times using a 95°C, 30:/62°C, 30:/72°C, 1′ profile, in a Perkin-Elmer 480, to yield a single product of the expected size. Triplicate PCRs were pooled and purified using the PCR Purification Spin Kit (Qiagen Inc., Studio City, CA) and cloned into the vector pCRII using the

TA Cloning Kit (Invitrogen, San Diego, CA). The resulting white colonies were used to prepare plasmid DNA for sequencing and plasmid construction. Restriction digestion indicated that the plasmids pα10/597 and pβ4/597 contained full-length PCR products of the TCR α and β genes respectively. These cloned PCR products were completely sequenced using SP6, T7, and the primers Mus Cαseq (5′-TCTCAGCTGGTACACG-3′; SEQ ID NO. 17) or Mus Cβseq (5′-CTGCTTCTGATGGCTCAAACACAG-3′; SEQ ID NO. 18), and Mus Cαf3 (5′-AAGCTTGTCTGGTTGCTCCA-3′; SEQ ID NO. 19) or Mus Cβf3 (5′-CTGCTCAGGCAGTAGCTATA-3′; SEQ ID NO. 20) using the Sequenase Kit (U.S.B., Cleveland, OH). The sequences of the TCR α and β genes contained in plasmids pα10/597 and pβ4/597 were found to be identical to the published sequences of the TCR genes of BDC 2.5 (Candeias *et al.*, *PNAS USA 88*:6167-70 (1991)).

Four baculovirus transfer vectors were constructed, two 'single promoter' vectors p3/598 and p7/599 (Figures 8 and 9) which contain either a BDC 2.5 β-chain-IgG1 TCR chimera or a BDC 2.5 α-chain-IgG1 TCR chimera respectively downstream of the polyhedrin promoter, and two 'dual promoter' vectors, p16/599 and p20/599 (Figures 10 and 11) both of which contain the BDC 2.5 TCR α-IgG1 and BDC 2.5 TCR β-IgG1 TCR chimeras (Figure 8). p3/598 was constructed by ligating into the BamHI site of pVL941 (Pharmingen, San Diego, CA) both a BglII to KpnI BDC 2.5 TCR β containing fragment from pβ4/597 and a KpnI to BglII IgG1 containing fragment from pIGG1 (1a)/584. Similarly p7/599 was constructed by ligating into the BamHI site of pVL941 (Pharmingen, San Diego, CA) both a BglII to KpnI BDC 2.5 TCR α containing fragment from pα10/597 and a KpnI to BglII IgG1 containing fragment from pIGG1(1a)/584. To construct the two dual transfer vectors first two plasmids, p5/598 and p7/598 were constructed, both of which contain the BDC 2.5 TCR β-IgG1 chimera downstream of either the polyhedrin

or P10 promoters respectively. p5/598 and p7/598 were constructed by ligating into either the BamHI or BglII sites of pAcUW51 (Pharmingen, San Diego, CA) both a BglII to KpnI BDC 2.5 TCR $\beta$ containing fragment from p$\beta$4/597 and a KpnI to BglII IgG1 containing fragment from pIGG1(1a)/584. Next, KpnI to PvuII and PvuII to BglII BDC 2.5 TCR $\alpha$ fragments from p$\alpha$10/597 and a BglII to HindIII BDC 2.5 TCR $\beta$-IgG1-containing fragment from p5/598 were ligated into p7/598 between the KpnI and HindIII sites to construct p16/599. Similarly, p20/599 was constructed by ligating between the EcoRI and KpnI sites of p5/598 an EcoRI to BamHI BDC 2.5 TCR $\beta$-IgG1 containing fragment from p7/598 and BglII to PvuII and PvuII to KpnI BDC 2.5 TCR $\alpha$ fragments from p$\alpha$10/597.

The baculovirus transfer vectors described above, p3/598, p7/599, p16/599 and p20/599 have been cotransfected into Sf9 cells along with linearized BaculoGold DNA (Pharmingen, San Diego, CA) following the manufacturers protocol, as described above. The infected cell supernatant contains a protein, as expected, that reacts with a V$\beta$4 specific mAb as a detection antibody in a sandwich ELISA that uses the mAb H57 (directed against C$\beta$) as a capture antibody in a protocol that is essentially identical to that described previously to assay soluble D10 dcTCR.

D. Synthesis of Vectors Encoding the BDC 6.9 TCR Genes as IgG1 Chimeras

Recombinant baculoviruses were constructed for expression of soluble BDC 6.9 TCR. As in the case of the BDC 2.5 TCR, described above, two viruses were designed to secrete the BDC 6.9 TCR as an IgG1 chimera. The genes for the $\alpha$ and $\beta$ chains of the BDC 6.9 TCR were amplified from RNA by PCR using conditions identical to those previously described above, except that RNA was prepared from BDC 6.9

T-cells (K. Haskins, Barbara Davis Center for Childhood Diabetes, Denver, CO) and different primer pairs were used in the PCRs. The 5′-primers used to amplify the α and β chains were 5′-CCTCTAGAAGATCTCCATGGGCTCCATTTTCCTCAGTT-3′ (Vβ4; SEQ ID NO. 21) and 5′-CCTCTAGAAGATCTTCATGAAAACATACGCTCCTACATTA-3′ (MusVα13.1; SEQ ID NO. 22), respectively, both of which are complementary to the 5′-end of the signal peptide sequence. Primers were designed based on published sequences. As the published sequence of the murine Vα13.1 gene segment is incomplete (Yague *et al.*, *Nuc. Acids Res.* *16*:11355-11364 (1988)) the 5′-terminal coding sequence needed to be determined. A plasmid, pBDC 6.9 α2.10/596, containing a PCR product generated by inverse PCR during the analysis of the BDC 6.9 TCR gene expression as described above, was found to contain the entire coding region of the Vα13.1 gene segment. The sequence of the 5′-end was determined (Figure 12; SEQ ID NO. 3 and 4) and the putative initiation codon of the signal sequence was determined based on published criteria (von Heijne, G. *Nuc. Acids Res.* *14*:4683-4690 (1986)).

The resulting PCR products were cloned into a plasmid vector, pCRII, as previously described above to yield four plasmids, pCR-BDC 6.9 α(Z), pCR-BDC 6.9 α(f4), pCR-BDC 6.9 β(Z), and pCR-BDC 6.9 β(f4). The TCR genes within these plasmids were sequenced as previously described above. pCR-BDC 6.9 α(Z) was found to contain two mutations, one of which introduced a stop codon within the coding sequence and another which altered a conserved cysteine codon to an arginine codon. pCR-BDC 6.9 α(f4) was found to contain one mutation in which the second stop codon had been altered to an arginine codon. To correct the detrimental mutations in pCR-BDC 6.9 α(Z), the nonmutated BstXI to AatII (TCR) gene fragment from

pCR-BDC 6.9 α(f4) was used to replace the corresponding fragment of pCR-BDC 6.9 α(Z) to yield p9/603. pCR-BDC 6.9 β(Z) was found to have a one-base pair deletion near the 5'-end of the TCR gene, which did not need to be corrected. pCR-BDC 6.9 β(f4) was found to contain no mutations.

In order to construct the pAcUW51-based plasmids containing genes encoding both the α- and β-chains of the BDC 6.9 TCR as fusions to the IgG1 CH2 and CH3 domains, two intermediate plasmids were first constructed. p51/604 was built by ligating both the KpnI to AgeI (IgG1-vector) fragment from p16/599 (Figure 10) and the SalI to KpnI (BDC 6.9 β) fragment from pCR-BDC 6.9 β(Z) between the SalI and AgeI sites of p16/599. p32/604 was built by ligating both the SalI to AgeI (BDC 2.5 β-vector) fragment from p7/598 and the SalI to KpnI (BDC 6.9 β) fragment from pCR-BDC 6.9 β(Z) between the KpnI and AgeI sites of p20/599. Next, p11/606 (Figure 13), containing the BDC 6.9 TCR β-IgG1 gene downstream of the polyhedrin promoter and the BDC 6.9 TCR α-IgG1 gene fragment from p9/603 between the KpnI and HindIII sites of p20/599. p33/606 (Figure 14), containing the BDC 6.9 TCR α-IgG1 gene downstream of the polyhedrin promoter and the BDC 6.9 TCR β-IgG1 gene downstream of the P10 promoter, was constructed by ligating both the KpnI to HindIII (IgG1) fragment from p20/599 and the BglII to KpnI (BDC 6.9 α) fragment from p9/603 between the BamHI and HindIII sites of p32/604.

E. Construction of Vectors Encoding α-chain TCR Genes with Carboxy Terminal Affinity Tails

Carboxy terminal extensions were attached to the α chain constant region to simplify the purification protocols. The ability of histidine to complex with metal was exploited (Hochuli *et al.*, *J. Chrom.* *411*:177-184 (1987)), as was the ability of a short peptide to bind to

streptavidin at the biotin binding site (Schmidt, T.G.M. and A. Skerra, *Prot. Eng.* 6:109-122 (1993)). The first ligand pair can be disrupted with imidazole, the second with biotin. Hexahistidine (HH) and StrepTag (ST) affinity tails were added to the 3'-end of the BDC 2.5 TCR α-IgG1 genes contained in both plasmids p16/599 and p20/599. To generate a hexahistidine coding sequence, with a 5' BstXI complementary end and a 3' BamHI complementary end, two complementary synthetic oligonucleotides, 5'-CTGGTAAACATCACCATCACCATCACTCACCCGGGAAGTAATGACTCGAG-3' (IGG1HHA; SEQ ID NO. 23) and 5'-GATCCTCGAGTCATTACTTCCCGGGTGAGTGATGGTGATGGTGATGTTTACCAGGAGA-3' (IGG1HHB; SEQ ID NO. 24), were annealed in a 50 μL volume containing approximately 5 μg of each by heating to 90°C for 15' and allowing the mixture to cool to room temperature. Similarly, a streptag coding sequence, with BstXI and BamHI complementary ends, was generated using oligonucleotides 5'-CTGGTAAAGCATGGCGACATCCGCAATTCGGGGGGTAATGACTCGAG-3' (IGG1STA; SEQ ID NO. 25) and 5'-GATCCTCGAGTCATTACCCCCCGAATTGCGGATGTCGCCATGCTTTACCAGGAGA-3' (IGG1STB; SEQ ID NO. 26). p11/607 (Figure 15), containing the BDC 2.5 TCR β-IgG1 gene downstream of the polyhedrin promoter and the BDC 2.5 TCR α-$IgG1_{HH}$ gene downstream of the P10 promoter was constructed by ligating both the SalI to BstXI (BDC 2.5 TCR α-IgG1) fragment from p16/599 and the BstXI to BamHI hexahistidine-encoding double stranded oligonucleotide between the SalI and BglII sites of p16/599. In an otherwise identical manner, p21/607 (Figure 16), was created by using the Streptag-encoding double-stranded oligonucleotide. p41/607 (Figure 17), containing the BDC 2.5 TCR β-IgG1 gene downstream of the P10 promoter and the BDC 2.5 TCR α-$IgG1_{HH}$ gene downstream of the polyhedrin promoter was constructed by ligating both the

SalI to BstXI (BDC 2.5 TCR α-IgG1) fragment from p20/599 and the BstXI to BamHI hexahistidine-encoding double stranded oligonucleotide between the SalI and BamHI sites of p7/598. In an otherwise identical manner, p51/607 (Figure 18), was created by using the Streptag-encoding double-stranded oligonucleotide.

Both the hexahistidine and streptag tails were also added behind the B10 TCR α-IgG1 gene contained in p2/246 (Figure 6). p11/608 (Figure 19), containing the B10 TCR α-$IgG1_{HH}$ gene behind the polyhedrin promoter and the B10 TCR β-IgG1 gene behind the P10 promoter, was constructed by ligating both the Xba to KpnI (B10 TCRα) fragment from p2/246 and the KpnI to HindIII ($IgG1_{HH}$) fragment from p41/607 between the XbaI and HindIII sites of p2/246. In an otherwise identical manner, p31/608 (Figure 20), containing the B10 TCR α-$IgG1_{ST}$ gene behind the polyhedrin promoter and the B10 TCR β-IgG1 gene behind the P10 promoter, was constructed by using the KpnI to HindIII ($IgG1_{ST}$) fragment from p51/607.

Two pVL941 based plasmids, p14/259 and p9/259 (Figures 23 and 24), containing either the hexahistidine or streptag affinity tails respectively, behind the D10 dcTCR α-gene were also constructed. A double-stranded oligonucleotide, encoding a hexahistidine sequence and two stop codons, and with AatII and BamHI complementary ends, was created by annealing two synthetic oligonucleotides, 5′-CCCCTGTCATCACCATCACCATCACTCACCCGGGAAGTAATGAGGTACCTCGAG-3′ (STHHA; SEQ ID NO. 27) and 5′-GATCCTCGAGGTACCTCATTACTTCCCGGGTGAGTGATGGTGATGGTGATGACAGGGGACGT-3′ (STHHB; SEQ ID NO. 28), under conditions identical to those described above. A similar oligonucleotide, encoding the streptag sequence and two stop codons, was created by annealing oligonucleotides 5′-CCCCTGTGCATGGCGACATCCGCAATTCGGGGGGTAATGAGGTACCTCGAG-3′

(STIIIA; SEQ ID NO. 29) and 5'-GATCCTCGAGGTACCTCATTACCCCCCGAATTGCGGATGTCGCCATGCACAGGGGACGT-3' (STIIIB; SEQ ID NO. 30). p14/259 was constructed by ligating into the dephosphorylated BamHI site of pVL941 both the BglII to AatII (D10 dcTCR α) fragment from p4/224 and the AatII to BglII hexahistidine-encoding double-stranded oligonucleotide just described. In an otherwise identical manner, p9/259 was constructed using the streptag-encoding oligonucleotide.

F. Accessibility of Thrombin Sites

A thrombin cleavage site was engineered between each TCR chain and the IgG1 domains in the D10-IgG1 TCR chimera. To determine the accessibility of these sites, 5 μg of purified D10 TCR-IgG1 chimera was digested with 2 μg of thrombin overnight at 37°C. Following digestion, the material was centrifuged at 4000 rpm for 10 minutes and the supernatant was subjected to SDS-PAGE under non-reducing conditions. An undigested control sample was prepared under the same conditions. Quantitative cleavage of the chimeric species to products of about 50 kDa, as predicted from the theoretical molecular weights of the TCR and IgG1 portions of the D10 TCR-IgG1 chimeric species, was observed. Despite the large ratio of thrombin to protein, there was no further proteolysis of the TCR. This indicates that both D10 dcTCR and IgG1 domains are folded in their respective native-like conformations.

EXAMPLE 4 Purification and Characterization of Expressed TCR

A. Immunoaffinity Purification and PAGE Analysis

An immunoaffinity matrix was prepared by covalently coupling 16 mg of 3D3 mAb to immobilized protein A beads (Repligen, Cambridge, MA) using dimethylpimelimidate as described (Harlow, E. and D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)). Supernatants from baculovirus-infected insect cell cultures were concentrated approximately five-fold using a Pellicon concentrator with a 10-kDa membrane filter (Millipore, Bedford, MA), and filtered through a 0.2 $\mu$ filter prior to passage over a 3D3 immunoaffinity column (XK16, Pharmacia, Piscataway, NJ). Typically, samples were applied overnight at 4°C with a flow rate of 1-2 ml/min. After washing the column with 10 column volumes of phosphate buffered saline (PBS: 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), bound material was eluted with 50 mM citrate (pH 3.0). Eluted fractions were neutralized with 1 M Tris-HCl (pH 8.8) and dialyzed against PBS. Protein concentrations were determined by the method of Bradford, using BSA as a standard (Bradford, M.M., *Anal. Biochem. 72*:248-254 (1976)). Material purified by immunoaffinity chromatography was analyzed by SDS-PAGE under non-reducing and reducing conditions.

Native PAGE and SDS-PAGE analyses were performed essentially as described (Laemmli, U.K., *Nature 227*: 680-685 (1970)). The nonreduced soluble D10 dcTCR migrated predominantly within the size range, Mr = 50-55 kDa (data not shown), which is in reasonable agreement with the calculated mass of 49.332 kDa. Since mAb 3D3 is specific for a conformational and clonotypic epitope of the D10 dcTCR formed by the juxtaposition of V$\alpha$ and V$\beta$ domains, the

immunoaffinity purified material appears to be heterodimeric and in the native conformation. After reduction, the protein migrated as a monomer with an Mr of approximately 30 kDa (data not shown), confirming that the dimer is linked by an intermolecular disulfide bond. In the nonreduced D10 dcTCR preparations, small amounts of covalently aggregated species, and a species with an Mr of 30 kDa can be observed (data not shown). Their presence varied from preparation to preparation, and never exceeded 10% of the total yield of immunoaffinity purified protein. The aggregated material does not appear to be a contaminant derived from the insect cell, nor does it appear to contain homodimers of $\alpha$- or $\beta$-chains. The 30 kDa Mr species could be derived from noncovalently linked heterodimers that dissociated in the presence of SDS.

Immunoaffinity purification of the D10 TCR-IgG1 chimera yielded material that migrated under nonreducing SDS-PAGE as two distinct species with Mr > 100 kDa (data not shown). Analysis by size exclusion chromatography indicated that the lower species represents heterodimeric D10 TCR-IgG1 with an Mr of approximately 120 kDa, whereas the upper species is covalently aggregated material. Upon reduction, each species migrated as a monomer of similar size (Mr = 50-55 kDa), indicating that both are held together by disulfide linkages. Unlike the D10 dcTCR construct, the purified D10 TCR-IgG1 chimera does not contain noncovalently linked $\alpha$- or $\beta$-chains.

To aid in characterization of D10 dcTCR, individual D10 $\alpha$ and D10 $\beta$ chains were purified from recombinant baculovirus-infected insect cell supernatants using H28 and H57 mAb immunoaffinity resins, respectively. SDS-PAGE analysis indicated the presence of monomeric and disulfide-linked polymeric species in both preparations (data not shown). The presence of unpaired cysteines in each of the chains (see Figures 1 and 2) cause the disulfide-linked

aggregation. The α-chain appeared to be significantly more aggregated than the β-chain.

Two-dimensional SDS-PAGE was performed on purified D10 TCR-IgG1, D10 dcTCR, and the individual α- and β- chains of the D10 dcTCR, under both equilibrium and non-equilibrium IEF conditions.

For purified D10 TCR-IgG1, analysis was carried out using equilibrium pH gradient electrophoresis in the first, and SDS-PAGE in the second dimension, as recommended by the manufacturer (BioRad, Hercules, CA). Two distinct, closely spaced spots, each migrating around 50 kDa in a neutral pH range, were observed (data not shown). The theoretical molecular masses and pI of D10 TCR α-IgG1 are 47.985 kDa and 6.0, and those of D10 TCR β-IgG1 are 51.672 kDa and 7.4, respectively. Although the two chains were not fully resolved, the results are consistent with the SDS-PAGE and IEF analyses, and further confirm the presence of α- and β-chains in the D10 TCR-IgG1 chimera.

Two sets of IEF conditions were used to separate the α- and β- chains of purified D10 dcTCR, because of difficulties in forming a linear 3-10 pH gradient on a single gel. Using equilibrium IEF under denaturing and reducing conditions in the first dimension, D10 dcTCR migrated as a single heterogeneous species with a molecular mass of about 30 kDa and an acidic pI (data not shown). This corresponds to the mobility of isolated D10 α chain analyzed under identical conditions (data not shown). Under the same conditions, the β-chain, which has a pI in the range of 8.6-8.8, could not be focused. When two-dimensional analysis was performed using non-equilibrium pH gradient electrophoresis (NEPHGE) under denaturing and reducing conditions in the first dimension, D10 dcTCR migrated as a single heterogeneous species with a similar molecular mass of about 30 kDa and a basic pI (data not shown). Based on the NEPHGE analyses carried out on

purified individual α- and β-chains D10 dcTCRs, this species corresponds to the β-chain. Under these conditions, the α-chain, which has a pI in the range of 4.8-5.0, failed to focus. The two-dimensional standards provided by the manufacturer (BioRad, Hercules, CA) also behaved similarly in both formats (data not shown). The data conclusively shown the presence of heterodimeric material in the affinity purified preparations of soluble D10 dcTCR.

B. Western Blot Analysis

The reactivity of Vα2-specific mAb to purified D10 dcTCR and D10 TCR-IgG1 was investigated. Protein samples subjected to SDS-PAGE under nonreducing or reducing conditions were electroblotted onto PVDF membranes using a semi-dry blotting system (Hoefer, San Francisco, CA). Unbound sites were blocked with blocking buffer (3% nonfat dry milk, 0.02% Tween-20 in PBS) and blots were incubated with an anti-Vα2 mAb at 1.5 μg/ml in blocking buffer overnight at room temperature. After washing with PBS, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2,000 dilution in PBS) for one hour. The membranes were washed extensively with PBS, and bound antibodies were detected by the ECL technique according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Vα2 mAb can recognize the α chain of each construct after exposure to SDS and subsequent transfer to PVDF membrane (data not shown), but not after reduction of disulfide bonds under otherwise similar conditions (data not shown). Thus, it appears that the integrity of the intramolecular disulfide bond in the vicinity of the variable region of the α-chain is critical for formation of the epitope recognized by the Vα2-specific mAb. The epitope does, however, appear to be resistant to SDS, at least under these assay conditions.

C. Immunoprecipitation

To investigate the presence of α- and β-chains in the immunoaffinity purified material, D10 dcTCR and D10 TCR-IgG1 preparations were immunoprecipitated with H28 and H57 mAb coupled to immobilized protein A beads. Immunoprecipitation of heterodimeric D10 dcTCR and D10 TCR-IgG1 chimera was performed as previously described (Harlow, E. and D. Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1988)). Briefly, culture supernatant or purified TCR were incubated overnight at 4°C with mAbs H28 (anti-Cα) or H57 (anti-Cβ) coupled to immobilized protein A beads. Immunoprecipitates were extensively washed with Tris buffered saline (TBS: 10 mM Tris-HCl, 0.9% NaCl, pH 7.4) containing 0.5% Triton X-100, and subjected to 12.5% SDS-PAGE under reducing conditions.

The purified proteins contained both α- and β-chains; moreover, both chains appeared to be present in equivalent amounts. These results were confirmed by nonreducing SDS-PAGE on immunoprecipitated samples (data not shown). For D10 dcTCR-containing cell culture supernatants, the immunoprecipitated material obtained with both antibodies was heterogeneous (Mr = 30-35 kDa) under reducing SDS-PAGE conditions (data not shown). The size range is consistent with the calculated values of molecular mass for the truncated D10 α- and β-chains (22.758 and 26.574 kDa, respectively). The higher Mr and the heterogeneity seem to be due to N-linked glycosylation.

Similar data as those obtained for soluble D10 dcTCR were obtained by SDS-PAGE analysis of immunoprecipitates of supernatants from Sf9 cell cultures expressing the D10 TCR-IgG1 chimera (data not shown). Both antibodies precipitated species of Mr = 50-55 kDa. the theoretical molecular masses of the chimeric D10 TCR α chain-IgG1 and

D10 TCR β chain-IgG1 are 47.984 and 51.671 kDa, respectively.

D. Conformationally-Sensitive Slot Blot Assay

1. D10 dcTCR

The presence of native-like material in the purified preparations of D10 dcTCR was confirmed by performing conformationally-sensitive ECL slot-blot analysis utilizing Vα2- and Vβ8- specific mAbs. Purified D10 dcTCR and D10 TCR-IgG1 were serially diluted (1 μg-8 ng) in PBS (pH 7.4) and applied to an Immunodyne activated membrane (Pall BioSupport Corporation, East Hills, NY) using a BioRad slot blot apparatus. After blocking with 3% nonfat dry milk in PBS, the membranes were incubated with various monoclonal antibodies for one hour at room temperature. After washing with PBS, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000 dilution) for one hour. The membranes were then washed extensively with PBS and developed using the ECL technique according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Supernatants from Sf9 cells infected with v9/237 (D10 dcTCR) showed reactivity, whereas those from cells independently infected with either v1/258 (D10 dcTCR α-chain) or v7/258 (D10 dcTCR β-chain) did not. Conformationally-sensitive ECL-slot blot assay using mAb 3D3 also confirmed the finding of the presence of native-like material in the purified preparations. Monoclonal antibody 3D3 did not react with the isolated individual α- and β- chains of D10 dcTCR (data not shown).

2. B10 TCR-IgG1 Chimera

RR8/H57/8G2 affinity purified B10 TCR-IgG1 chimera was subjected to a conformationally-sensitive ECL assay as described previously. The results indicated that the eluted material shows strong reactivity to all three

monoclonal antibodies 8G2, H57, and H28, further indicating the presence of correctly folded heterodimeric B10 TCR-IgG1 in the IA eluted fractions (data not shown).

E. ELISA for Soluble TCR

Two sandwich ELISAs were developed to assay the concentration of D10 dcTCR and D10 TCR-IgG1 proteins in the media of recombinant baculovirus infected cell cultures. One assay, called 3D3/V$\beta$8, is specific for the D10 dcTCR. It used the D10 dcTCR clonotype-specific mAb 3D3 adsorbed to plastic as the capture antibody, and the V$\beta$8-specific mAb as the detecting antibody. Another assay, termed H57/H28, used the C$\beta$-specific mAb H57 to capture, and the anti-C$\alpha$ mAb H28 to detect the soluble TCR.

Ninety-six well Maxisorp Immunomodules (Nunc, Naperville, IL) were coated with capture antibody by overnight incubation at 4°C with 100 $\mu$l per well of antibody (4 $\mu$g/ml) in borate saline buffer (BSB:100 mM $H_3BO_3$, 75 mM $Na_2B_4O_7$, 100 mm NaCl, pH 8.3). Plates were washed six times with BSB-0.05% Tween-20 afterwards, and between each subsequent step. Plates were blocked for four hours by incubation at room temperature with BSB-1%BSA. Serially diluted standards and samples of soluble TCR preparations in BSB-1% BSA were added in duplicate. After overnight incubation at 4°C, 0.2 $\mu$g biotinylated detecting antibody was added, and incubation continued for a further four hours. Following standard protocols, plates were then incubated for one hour with 20 $\mu$g of streptavidin-labeled alkaline phosphatase (Kierkegaard and Perry Labs, Gaithersberg, MD), and developed with Sigma 104 phosphatase substrate (Sigma, St. Louis, MO). Absorbance was measured at 405 nm.

F. Isoelectric Focusing (IEF)

The presence of $\alpha/\beta$ heterodimer in affinity-purified material was further confirmed by isoelectric focusing analyses under non-denaturing conditions. IEF was performed using Servalyt precoat gels (pH 3-10) under native conditions at 4°C as described (Khandekar, S.S., *et al.*, *Protein Expression and Purification* 4:580-584 (1993)). For D10 dcTCR, isoelectric points (pIs) of 6.4-6.6 for the heterodimer, and 4.8-5,0 and 8.45-8.65 for the individual $\alpha$- and $\beta$- chains were observed (data not shown). The pIs predicted from the primary amino acid sequence are 4.9 for the $\alpha$ chain and 8.0 for the $\beta$ chain. Thus, the theoretical pI for the heterodimer is 6.45. These values appear to be in close agreement with those observed, and further confirm that 3D3 immunoaffinity purified preparations of D10 dcTCR are heterodimeric. Purified D10 TCR-IgG1 chimera under nondenaturing conditions were observed to have a pI of 6-6.5 (data not shown). This is in close agreement to the value of 6.5 predicted from the primary sequence of the D10 TCR-IgG1 chimera. Importantly, species with the pI values predicted for $\alpha\alpha$ or $\beta\beta$ homodimers were not observed. These results demonstrate the absence of homodimeric material in the 3D3 IA purified preparations of D10 dcTCR and D10 TCR-IgG1.

G. Size Exclusion Chromatography (SEC)

The apparent native molecular weights of purified D10 dcTCR and D10 TCR-IgG1 were determined by chromatography on a Superdex 200 PG 10/30 size exclusion column (Pharmacia, Piscataway, NJ) equilibrated with PBS (pH 7.4) and calibrated with the following molecular mass standards: thyroglobulin (666 kDa), $\gamma$-globulin (158 kDa), ovalbumin (43 kDa), myoglobin (17 kDa), and vitamin B12 (1 kDa). A 200 $\mu$g aliquot of purified protein was injected and eluted

at a flow rate of 0.5 ml/min, and 0.5-ml fractions were collected.

The native molecular mass of purified soluble D10 dcTCR was determined to be approximately 58 kDa (data not shown). This value is higher than that predicted from the primary sequence of D10 dcTCR, and this appears to be due to glycosylation of the protein in insect cells. When purified D10 TCR-IgG1 chimera were subjected to size exclusion chromatography, the dimeric species (Mr = 120 kDa) was separated from the polymeric, aggregated species. The aggregation seems to occur primarily via covalent interactions, because upon reduction, these species migrate as monomeric species in the size range of Mr = 50-55 kDa.

H. N-glycanase Treatment

SDS-PAGE, IEF, and SEC analyses implied that D10 dcTCR purified from recombinant baculovirus infected cell supernatant was heterogeneous in charge and size. Since the heterogeneity could result from differential glycosylation of the $\alpha$- and $\beta$-chains, the affinity purified D10 dcTCR was treated with N-glycanase under denaturing and reducing conditions so as to release all common classes of Asn-linked oligosaccharides (Maley, F. *et al.*, *Anal. Biochem. 180*:195-204 (1989)). N-glycanase digestions were performed according to the manufacturer's instructions (Genzyme Corp., Framingham, MA). Briefly, 50 $\mu$g of purified D10 dcTCR was incubated in 40 mM sodium phosphate buffer (pH 8.0) containing 0.5% SDS, 50 mM 2-mercaptoethanol and 1.5% NP40. The sample was boiled for five minutes to fully denature the protein, which was then incubated in the presence of N-glycanase at 37°C, and analyzed by SDS-PAGE.

The results indicated that treatment with N-glycanase reduced both the size and the heterogeneity (data not shown), indicating the presence of N-linked glycosylation

sites in D10 dcTCR. Although we have not yet identified these sites, inspection of the primary amino acid sequence (Figures 1 and 2) of the receptor reveals three potential Asn-linked sites in the α- chain and six in the β-chain.

I. Surface Plasmon Resonance (SPR) Binding

The specificity of mAb 3D3 for D10 dcTCR and D10 TCR-IgG1 chimera was evaluated using the BIAcore™ biosensor system (Pharmacia, Piscataway, NJ). The technique can detect binding of soluble analytes to a ligand immobilized on a dextran-coated chip in real time (Fagerstam, L., *Tech. Prot. Chem. 2*:65-71 (1991); Malmqvist, M., *Current Biology 5*:282-286 (1993)). For binding experiments, 0.6 μg of pure mAb 3D3 was coupled to dextran surface by standard amine chemistry (Johnsson, B. *et al.*, *Anal. Biochem. 198*:268-277 (1991)). The immobilization level was 2335 RU. Throughout the binding experiments, a flow of HEPES buffered saline (HBS: 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.5% surfactant P20, pH 7.4) at 5 μl/min was maintained. Soluble two-domain CD4 (provided by M. van Schravendijk, Procept, Inc., Cambridge, MA) and BDC 6.9 TCR- and BDC 2.5 TCR-IgG1 chimera proteins (McKeever, *et al.*, in preparation) were used as specificity controls. Samples of 20 μl were injected at a flow rate of 5 μl/min. At the end of each binding cycle, the biosensor surface was regenerated with 10 mM HCl. All binding experiments were conducted at 25°C. The SPR signal was recorded as RU versus time and was plotted as a "sensogram". The data were analyzed according to the manufacturer's instructions (Pharmacia, Piscataway, NJ). An increase in RU indicates binding of injected analyte to ligand immobilized on the surface of the biosensor chip.

D10 dcTCR at 0.3 μM gave a significant binding signal (1384), indicating the formation of the binary complex

(data not shown). This signal was reproducible (data not shown). In control experiments, D10 dcTCR did not bind to the dextran matrix alone (data not shown). Binding of the purified α- and purified β-chain of D10 to immobilized 3D3 was also monitored. Neither purified D10 dcTCR α- nor β-chains at 1.5 μM and 3 μM bound to immobilized 3D3. These concentrations were in 5- and 10-fold excess of that of the D10 dcTCR.

The clone specific characteristics of the mAb 3D3 were further examined by analyzing the B10 TCR-IgG1, 2-domain CD4, and BDC 6.9 TCR. None of these proteins showed any binding to the immobilized 3D3. These experiments confirm the anticlonotypic properties of mAb 3D3 for D10 dcTCR. The results also show that purified D10 and D10 TCR-IgG1 preparations are heterodimeric, since purified α and β chains did not interact with the immobilized 3D3.

## EXAMPLE 5 dcTCRs Can Immunize Syngeneic Mice to Produce a Specific Antibody Response

### A. Materials

*Animals*

NOD mice were purchased from Taconic Farms (Germantown, NY) and were housed in a conventional animal facility. The cumulative incident of diabetes at 27 weeks was 70-80% for females and 40-50% for males. NOD/Lt-Tg(RIPTag)1Lt (hereafter referred to as NOD/Lt RIP-Tag) transgenic mice, described by Hamaguchi *et al.* (*Diabetes 40*:842 (1991)) were bred in our colony from breeding pairs obtained from Dr. E. Leiter of The Jackson Laboratory. These mice subsequently became available from the Animal Resources Unit of The Jackson Laboratory. The transgene is a recombinant simian virus 40 (SV40) oncogene in which the rat insulin 5′ promoter has been inserted immediately upstream of the SV40 early region (Hanahan, D., *Nature*

315:115 (1985)); consequently, transgene expression is restricted to the β cell, resulting in the transformation of this cell type exclusively. Thus, the animals develop β cell tumors at 10-16 weeks of age which cause hypoglycemia resulting in rapid onset of death. The first litters from these mice were perpetuated by brother-sister mating. Later, in order to avoid the loss of litters due to death of mothers that succumb to tumors before their offspring are weaned, transgenic males were mated to NOD females. After weaning, the young animals were provided with 5% (w/v) sucrose supplemented water, and 3 weeks later, sugar cubes were added. From about 10 weeks of age, blood glucose levels were closely monitored using a glucose II analyzer, (Beckman Instruments, Inc. Somerset, NJ), and when the mice became severely hypoglycemic (serum glucose concentration ≤ 50 mg/dl) they were killed for islet cell preparation.

*Antigen Preparations*

Islet cells were isolated from NOD mice according to methods outlined previously (Haskins, K., *et al.*,*Diabetes* 37:1444-1448 (1988); Haskins, K. *et al.*,*Proc. Natl. Acad. Sci. USA* 86:8000-8004 (1989)) apart from the following modifications: after the islets were hand-picked from the exocrine tissue they were then put through a 70-μm nylon mesh screen (cell strainer, Falcon, Oxnard, CA) without any further digestion. This cell suspension was then frozen in liquid nitrogen until required. Before use as islet antigen in proliferation assays or for propagation of cell lines, cells were rapidly thawed at 37°C. To prevent the incorporation of $^{3}$H-thymidine by tumor cells or by T-cells in islet infiltrates all pancreata from tumor and non tumor bearing mice were irradiated with at least 4000R prior to the collagenase digestion step. Islet tumors were

harvested from tumor-bearing NOD/Lt Rip-Tag or (NOD x NOD/Lt RIP-Tag)F1 pancreata by the method of Bergman and Haskins (*Diabetes 43*:197 (1994)) with the modification that immediately after death the pancreas was infused with a collagenase (Boehringer Mannheim, Indianapolis, Indiana) solution (0.2 mg/ml) via the common bile duct. The pancreas was excised and incubated in vitro for 15 min. at 37°C. The encapsulated tumors were dissected from exocrine tissue and forced through a 70-μm nylon mesh screen to produce a single cell suspension. The cells were examined microscopically to ensure viability prior to freezing at -70°C. Tumor size varies but can yield approximately twenty times the number of cells that can normally be isolated from the pancreas of a NOD mouse. NOD/Lt RIP-Tag and (NOD x NOD/Lt RIP-Tag)F1 islet cells prepared in this way, when compared with equivalent numbers of normal NOD mouse islet cells, appear to be highly enriched for the antigen which stimulates the proliferation of BDC 2.5 and 6.9 T cells clones *in vitro* in the presence of NOD antigen presenting cells (U. McKeever, unpublished observations).

*T Cell Clones*

The I-$A^{g7}$-restricted, $CD4^+$, islet antigen specific T-cell clones have been described (Haskins, K., *et al.*,*Diabetes 37*:1444-1448 (1988); Haskins, K. *et al.*,*Proc. Natl. Acad. Sci. USA 86*:8000-8004 (1989)). They were derived from spleen and lymph node preparations from newly diabetic, 3- to 5-month old mice and have been carried continuously on syngeneic islet cells and antigen presenting cells. Both BDC 2.5 and BDC 6.9 have been demonstrated to be diabetogenic upon transfer into nonirradiated very young NOD mice. Based on their cytokine profile (IL2, γIFN and TNF), they both belong to the Th1 subset.

The B10 T cell clone ($IE^k$ restricted, cytochrome C peptide specific) was provided by Stephen Hedrick and Gerald Siu (University of California, San Diego, CA). The D10.G4.1 AKR mouse T cell clone ($IA^k$ restricted, hen egg conalbumin peptide specific) was obtained from ATCC, Rockville, MD.

*Immunofluorescence staining*

Staining buffer for immunofluorescence was prepared by supplementing PBS-D with 0.1% sodium azide and 5% FCS. For direct immunofluorescence analysis, a total of 0.5 x $10^6$ to 1 x $10^6$ cells were resuspended in 10 $\mu$l of the appropriate dilution of FITC-conjugated, biotin conjugated or PE conjugated antibody in staining buffer in 96 well U-bottom microtiter plates, and incubated for 30 min. at 4°C. FITC-streptavidin (Pierce, Rockford, IL) was added where needed and plates were incubated for another 30 min. The cells were washed twice and finally resuspended in PBS containing 1% paraformaldehyde as previously described (Jones, B. *et al.*, *J. Immunol. 136*:348-356 (1986)). The number of cells analyzed by flow cytometry using a FACScan (Becton Dickinson, CA) varied from 5000 to 30000. Cells were gated according to forward and side scatter parameters.

For indirect immunofluorescence analysis of serum antibodies 1 x $10^6$ T cells were incubated (30 minutes at 4°C) with 1:10, 1:100 and 1:1000 dilutions of normal mouse serum or the antisera, washed three times by centrifugation, and incubated for a further 30-minute period with 10 $\mu$l of the appropriate dilution of an F(ab')2 fragment of fluorescein-conjugated goat anti-mouse IgG (whole molecule) (Organon Teknika Corporation, Cappel™, Durham, NC). After three washes to remove unbound antibody, the cells were fixed and analyzed on the FACScan as described above. Controls routinely included the FITC

conjugate alone or irrelevant rat and mouse mAb substituted for the primary antibodies.

The NOD mouse T cells were purified by applying a whole spleen cell population to T cell columns according to the manufacturers' instructions (R & D Systems, Minneapolis, MN).

*Proliferation Assay*

T cell proliferation assays were performed in Click's medium (EHAA: Irvine Scientific, Santa Ana, CA) supplemented with 5% FCS (Hyclone Labs, Logan, UT). Cultures were set up in triplicate in 200 µl volumes in 96-well round-bottomed plates. Irradiated (3000R) syngeneic mouse splenocytes were routinely used as antigen presenting cells (APCs). T cell proliferation was assessed by pulsing each culture with 1 µCi $^{3}$H-thymidine for the final 12-16 hours of incubation. The cells were harvested on a Tomtec harvester (Tomtec, Orange, CT) and radioisotope incorporation measured using a beta-plate scintillation counter (Wallac, Gaithersburg, MD).

B. The dc BDC 2.5 TCR-IgG1 Stimulates an Antibody Response in the NOD Mouse

NOD mice were primed with the BDC 2.5 TCR-IgG1 protein in complete Freund's adjuvant. Boosting injections without adjuvant were given 14-21 days after priming and repeated after a further 7-14 days. Indirect immunofluorescence with sera collected 4 days after the second boosting injections revealed that 18 out of 32 immunized animals made antibodies that bound the surface of the BDC 2.5 T cell clone. Positive antisera were pooled and further analysis revealed that the antibodies were apparently specific for the BDC 2.5 clone. Typical data are shown in Figure 25(A). The antiserum stained BDC 2.5 T cells but

not BDC 6.9 or normal splenic NOD T cells, suggesting that the antibodies recognized clonotypic epitopes of the BDC 2.5 TCR. Specificity for BDC 2.5 versus 6.9 cells was also observed with each of the 14 mouse sera that were tested individually. Detailed mapping of these epitopes in the variable region of the BDC 2.5 TCR was not possible due to the lack of mAb specific for this receptor; however, some association with the $V_\beta$-4 segment was indicated by the ability of the antiserum to inhibit the binding of $V_\beta$-4 family-specific FITC-mAb. Data obtained with the pooled antisera are shown in Figure 25(B), and similar inhibition was seen with antiserum samples from individual mice (data not shown). As expected, given the apparent clonotype-specificity of the antisera (Figure 25(A)), it did not interfere with the binding of the FITC-mAb, H57, which recognizes an epitope of the Cβ-region, to any greater extent than unimmunized NOD mouse serum.

Antibodies recognizing TCR variable region epitopes have been found to inhibit the antigen-specific responses of T cell clones *in vitro* (White, J. et *al.*, *J. Immunol. 130(3)*:1033-1037 (1983)). The inhibitory activity of the BDC 2.5 specific antiserum was investigated in *in vitro* cultures of BDC 2.5 or 6.9 cells, stimulated by irradiated NOD mouse spleen cells and pancreatic islet cells as sources of APC and antigen. BDC 2.5 or 6.9 rested T cells were preincubated with or without the indicated dilutions of antisera raised against BDC 2.5 TCR-IgG1 or D10 dcTCR proteins, and then tested for their response to NOD/RIP-Tag islet cells and NOD APC in the standard proliferation assay. The anti-D10 dcTCR antiserum contained antibodies specific for the D10 T cell clone, and was used as a control. Unstimulated control cultures received APC without islet cells. The data are shown in Figure 26. Values represent the mean $^3$H-thymidine incorporated during

a 15 hr. pulse after a 72 hr. culture period. The data of Figure 26 demonstrate specific inhibition of the proliferative response of the BDC 2.5 cells, and therefore support the conclusion that NOD mouse antiserum raised against the soluble BDC 2.5 TCR-IgG1 protein contains antibodies specific for clonotypic epitopes of the BDC 2.5 cell surface TCR.

## EXAMPLE 6 BDC 2.5 Clonotype-Specific Antibodies Produced in Response to Immunization of NOD Mice with BDC 2.5 TCR-IgG1 are Maternally Transferred and Protect Against Adoptively Transferred Diabetes

### A. Materials

*Animals*

The source and characteristics of NOD mice are described above under Example 5A. AKR/J mice were purchased from Jackson Laboratories (Bar Harbor, ME), housed in a conventional animal facility and used between 6 and 8 weeks of age.

*T cell clones*

The pancreatic β-cell specific NOD T cell clones, BDC 2.5 and BDC 6.9 are described above under Example 5A. The I-$A^k$-restricted, CD4+ hen-egg conalbumin peptide specific T cell clone D10 has been described (Kaye, J., *et al., J. Exp. Med. 158*:836-856 (1983)). It was derived from the lymph nodes of conalbumin immunized AKR mice. B1 was derived by a similar procedure at Procept from the lymph nodes of NOD mice and is an autoreactive (anti-I-$A^{g7}$) CD4+ T cell clone.

*Purification of spleen T cells*

Red cells were removed from the spleen cell population and the T cells isolated by negative selection on mouse T cell enrichment columns (R & D Systems, Minneapolis, MN) according to the manufacturer's instructions.

*Adoptive transfer of diabetes*

The procedure used was essentially that described by Haskins and McDuffie (*Science 249*:1434-1436 (1990)). NOD pups of 2-3 weeks of age were injected intraperitoneally with 5 x $10^6$ to 10 x $10^6$ of BDC 2.5 or 6.9 cloned T cells described under Example 5A. After one week a second injection of T cells was given followed by a third injection one week later. The animals were weaned at 3 weeks of age. The onset of diabetes was monitored by investigating the urinary glucose level with Test tape (Lilly, IN) and disease was confirmed by measuring the blood glucose levels with a Glucose Analyzer 2 (Beckman, CA). Diabetes was indicated by a blood glucose concentration of >10 mM.

B. <u>The Maternal Transfer of Anti-BDC 2.5 TCR Clonotype Antibodies Induced by Immunization with BDC 2.5 TCR-IgG1 Protects Young NOD Mice from Diabetes Resulting From the Adoptive Transfer of the BDC 2.5 T Cell Clone</u>

One group of 8-10 week old female NOD mice was immunized with BDC 2.5 TCR-IgG1 protein as described previously (Example 5B). A second group was immunized in a similar fashion with the D10 TCR-IgG1 protein, and a third group received no immunization. All the mice were mated with 8-10 week old male NOD mice. In the immunized groups mating began on the day of the first boosting injection, and pregnant mice were further boosted by the intravenous injection of 20 μg TCR-IgG1 at approximately day 13-16 of

gestation. Nursing mothers were given final boosts 14 days later. Indirect immunofluorescence with sera collected 4 days after the final boosting injection was used to determine which BDC 2.5 TC-IgG1 immunized mothers had mounted a BDC 2.5 TCR clonotype-specific-response as described in Example 5B. All of the D10 TCR-IgG1 immunized mothers made antibodies specific for the cell surface clonotype of the D10 TCR, as indicated by immunofluorescent staining of D10 cells but not of B1 clone T cells which, like the D10 clone, utilize TCR V$\beta$-8.1/2 and V$\alpha$-2 segments.

Starting at 2 to 3 weeks of age, pups born of the immunized NOD mothers and a group born of non-immunized mothers were injected once per week for three weeks with 5 x $10^6$ to 10 x $10^6$ viable T cells of either the BDC 2.5 or BDC 6.9 islet-specific clones. The onset of diabetes was monitored, and the data are presented in Table II.

Table II. Incidence of diabetes in adoptively transferred litters born from soluble TCR immunized mothers

| Soluble TCR immunization of mother | Maternal antibodies produced | Incidence of diabetes in offspring injected with the diabetogenic T cell clone: | |
|---|---|---|---|
| | | BDC 2.5 | BDC 6.9 |
| non-immunized | none | 20/63 (32%) | 7/16 (44%) |
| BDC 2.5 TCR-IgG1 | BDC 2.5 clonotype-specific antibody positive | 0/21 (0%) | 4/10 (40%) |
| BDC 2.5 TCR-IgG1 | BDC 2.5 clonotype-specific antibody negative | 17/29 (59%) | 8/18 (44%) |
| D10 TCR-IgG1 | D10 clonotype-specific antibody positive | 6/28 (21%) | not tested |

As expected from previously published data (Haskins, K. and McDuffie, M., *Science 249*:1433-1436 (1990)) both BDC

2.5 and BDC 6.9 T cells induced the early onset of diabetes. Disease occurred between 10 to 25 days after the first injection of cells in 30-50% of pups delivered by non-immunized mothers, as opposed to the natural time of onset, which is usually at >3 months of age in unmanipulated NOD mice. Immunizations of mothers that successfully resulted in a BDC 2.5 TCR clonotype-specific antibody response appeared to completely protect their pups from the induction of diabetes by the adoptive transfer of the BDC 2.5 T cell clone. As described above in Example 5, the islet-specific BDC 6.9 T cell clone is clonotypically distinct from BDC 2.5. Injection of the BDC 6.9 clone into pups from mothers producing BDC 2.5 TCR clonotype-specific antibodies resulted in accelerated diabetes with an incidence similar to that in control pups from non-immunized mothers. The protection against adoptively transferred disease afforded by maternal immunization therefore appeared to be immunologically specific. The immunological specificity of the maternally transferred protection was further demonstrated by the failure of immunization against the D10 clonotype to provide protection. The BDC 2.5 TCR clonotype-specificity of the maternally-transferred protection strongly suggests that it was mediated via the transfer of specific antibodies from the mother, either transplacentally or in milk, or by both routes. That is, protection resulted from the transfer of TCR-specific antibodies with the properties described under Example 5B. This interpretation was supported by the demonstration that 3-4 week old pups delivered by mothers successfully immunized with TCR-IgG1 protein contained titers of clonotype-specific antibodies of >1/100. The data support the concept that immunization with soluble TCR can induce an immune response of therapeutic value in protection against autoimmune diseases caused by T cell

specifically recognizing tissue-specific autoantigens: in this example, a pancreatic islet β-cell antigen.

EXAMPLE 7 D10TCR-IgG1 Immunization Stimulates Production of Vβ-8 Family-Specific Antibodies that are Maternally Transferred and Remove Mature Vβ-8-Expressing T Cells from the Peripheral Immune System of the Mature Animal

A. Materials

*Animals*

AKR/J and SJL/J mice were purchased from Jackson Laboratories (Bar Harbor, ME), housed in a conventional animal facility and used between 6 and 10 weeks of age.

*T cell clones*

The I-$A^k$-restricted, CD4+ hen-egg conalbumin peptide specific T cell clone D10 has been described (Kaye, J., *et al., J. Exp. Med. 158*:836-856 (1983)). It was derived from the lymph nodes of conalbumin immunized AKR mice. B1 was derived by a similar procedure from the lymph nodes of NOD mice and is an autoreactive (anti-I-$A^{g7}$) CD4+ T cell clone.

*Antibodies*

Monoclonal antibodies (MAb) specific for mouse TCR Cβ, Vβ-8.1/2, and Vβ-8.3, were obtained from Pharmingen (San Diego, CA) as fluorescein conjugates.

*Spleen cell cultures*

5 x $10^6$ spleen cells were cultured in the wells of 24-well, flat-bottomed plates (Corning, Corning, NY) in 2 ml cultures in Click's medium (Irvine Scientific, Santa Ana, CA), supplemented with 5% fetal calf serum (Hyclone Laboratories, Logan, UT). Staphylococcal enterotoxin (SEB)

purchased from Toxin Technologies (Sarasota, FL) was added to the culture medium at a final concentration of 1 μg/ml. After 4 days of culture at 37°C in a humidified $CO_2$ incubator, recombinant human IL-2 (R&D Systems, Minneapolis, MN) at a concentration of 5 ng/ml was added. This step was repeated 2 days later and incubation continued for a further 3 days.

B. TCR Vβ-8 Family-Specific Antibodies are Produced in Response to Immunization of SJL Mice with D10 TCR-IgG1

In the human autoimmune diseases rheumatoid arthritis and multiple sclerosis it appears that the immunological destruction of the target organ involves the activation of T cells with TCR containing β-chain variable regions encoded by restricted sets of Vβ-gene segments (Colleen O., *Immunol. and Cell Biol.* 73:297-307 (1995)). Therapies designed to eliminate just those clones of T cells bearing the relevant Vβ segments could have a high therapeutic index in these diseases since the removal of T cells using only several of a total repertoire of 50-60 different Vβ gene segments (Hali *et al.*, *Eur. J. Immunol.* 24:641-645) would not be expected to compromise overall immune responsiveness to environmental pathogens. Soluble TCR immunizations designed to induce the formation of Vβ family-specific antibodies provide one approach to Vβ-targeted therapy. The presence of Vβ family-specific antibodies *in vivo* should deplete the pool of mature T cells bearing members of the Vβ family recognized by the antibodies, while T cells expressing unrelated Vβ segments should be unaffected. The allele of the SJL mouse inbred strain has deleted Vβ 5, Vβ 8, Vβ 9, Vβ 11, Vβ 12, and Vβ 13 structural genes; consequently, mice of this strain are not tolerant to Vβ-8 antigenic epitopes. Immunization of C57L/J mice, which have a similar deletion of Vβ genes,

with Vβ-8-expressing T cells have been shown to stimulate the production of Vβ-8 family-specific antibodies (Staerz *et al., J. Immunol. 134*:3994-4000). Immunizations of SJL mice with soluble D10 TCR have been performed in order to investigate the immunotherapeutic potential of an antibody response specifically directed towards a particular TCR variable region family.

The D10 TCR contains a Vβ-segment encoded by a member of the Vβ-8.2 gene family. The ability of immunizations with the D10 TCR-IgG1 protein to stimulate an antibody response specific for TCR Vβ-8 segments was tested by immunizing SJL mice. SJL mice were each primed by subcutaneous injection in the hind limbs with 20-25 μg D10 TCR-IgG1 protein emulsified in complete Freund's adjuvant. Intraperitoneal boosting injections without adjuvant were administered 14-21 days after priming, and repeated after a further 7-14 days. Indirect immunofluorescent staining of the D10 cells surface with sera collected 4 days after the last injections demonstrated the production of antibodies recognizing the D10 TCR. Further analysis demonstrate that the serum antibody response was against Vβ-8.1/2 family epitopes. The data are shown in Figure 27. The antiserum stained the B1 T cell clone, which like the D10 clone expresses a TCR containing a Vβ-8.1/2 segment, but has a different clonotype. Mice in which the Vβ-8 structural genes are intact can contain up to 40% mature peripheral T cells with TCR containing Vβ-8.1/2 (Staerz *et al., J. Immunol., 134*:3994-4000). By indirect immunofluorescence, 9% of AKR mouse splenic T cells reacted with SJL mouse antiserum raised against D10 TCR-IgG1, thereby supporting the Vβ-8 specificity of the serum antibodies. The presence of a comparatively low proportion of Vβ-8-expressing T cells in AKR mice is probably a result of the deletion of Vβ-8.1 expressing T cells by superantigen coded by the Mtv.7 provirus in this strain (Hodes, R.J. and Abe, R. In

*Current Protocols of Immunology*, John Wiley and Sons, NY, pp. A.1.2.1-A.1.2.5).

C. Maternal Transfer of Vβ-8 Family-Specific Antibodies Suppresses the Development of Mature, Functional Vβ-8-Expressing Peripheral T Cells

In order to investigate the *in vivo* effects of Vβ-8-specific antibodies induced by immunization with soluble D10 TCR-IgG1, female SJL mice of 8 to 10 weeks of age were immunized with D10 TCR-IgG1 or BDC 2.5-TCR IgG1. Female AKR mice were immunized with D10 TCR-IgG1. The immunizations were performed as described in Example 7B immediately above. The female SJL mice were mated with unimmunized SKR mice and the female AKR mice with unimmunized SJL mice. The mating pairs were set up on the day of the first boosting injection. A group of age matched, unimmunized female SJL mice were similarly mated with unimmunized AKR mice. Immunized pregnant animals were boosted with 20 μg of the appropriate soluble TCR dissolved in PBS. D10 TCR-IgG1 immunization of the SJL mothers resulted in the reproducible transfer of antibodies recognizing Vβ-8.1/2 family-specific antigens as demonstrated by indirect immunofluorescent cell-surface staining of both D10 and B1 cells with sera collected from the (SJL x AKR)F1 offspring. The D10 clone from which the D10 TCR was originally produced was derived from AKR mice (Kaye, J., *et al.*, *J. Exp. Med.* 158:836-856 (1983)). Since AKR mice possess Vβ-8 family structural genes, they are tolerant of Vβ-8.1/2 family-specific epitopes. Therefore, in this mouse strain, D10 TCR-IgG1 immunization does not stimulate the production of antibodies recognizing these antigens. Instead, the antisera collected from AKR mothers were specific for D10 clonotypic epitopes, as indicated by indirect immunofluorescent staining of D10 cells but not of the B1 cloned T cells, which, similarly to the D10 clone,

use TCR Vβ-8.1/2 and Vα-2 genes. The clonotype-specific antibodies were also transferred to the (AKR x SJL)F1 offspring. The data are presented in Table III.

Table III Maternal Transfer of Vβ-8.1/2 Family-Specific Antibodies From Immunized SJL Mother to Their (SJL x AKR)F1 offspring

| Soluble TCR immunization of mother | Mating: (female x male) | Maternal antibodies produced | Titer of anti-Vβ-8.1/.2 antibodies in (SJL x AKR) F1 offspring |
|---|---|---|---|
| None | SJL x SKR | None | No antibodies |
| D10 TCR-IgG1 | SJL x AKR | anti-Vβ-8.1/.2 | >1/100 |
| BDC 2.5 TCR-IgG1 | SJL x AKR | anti-BDC 2.5 clonotype | >1/100 |
| D10 TCR-IgG1 | AKR x SJL | anti-D10 clonotype | >1/100 |

As described above, SJL mice were congenitally unable to express TCR Vβ-gene segments of the Vβ-B family; but normally, F1 animals produced by crossing SJL with AKR mice do make TCR containing Vβ-8 segments due to the introduction of intact Vβ-8 structural genes from the AKR genome. In the offspring of the various crosses involving either unimmunized mothers or mothers immunized against the soluble TCR, the purified splenic T cells were analyzed for the expression of Vβ-8 genes by cell surface immunofluorescence. The data are presented in Table IV.

Table IV Maternal Immunization Against TCR Vβ-8 Family Epitopes Specifically Depletes the Peripheral T Cell Population of T Cells Utilizing Vβ-8 Gene Segments

| Soluble TCR immunization of mother | Mating: (female x male) | F1 antiserum specificity | %[1] of purified[2] splenic T cells expressing Vβ-8 segments in F1 mice at age: | | | |
|---|---|---|---|---|---|---|
| | | | 5-7 weeks | | 11-15 weeks | |
| | | | Vβ-8.2 | Vβ-8.3 | Vβ-8.2 | Vβ-8.3 |
| non-immunized | SJL x AKR | none | 6.8±0.4 (10) | 4.0±0.5 (10) | ND[3] | ND |
| BDC 2.5 TCR-IgG1 | SJL x AKR | Anti-BDC 2.5 clonotype | 5.9±0.3 (11) | 3.0±0.2 (11) | 7.4±0.3 (7) | 4.8±0.1 (7) |
| D10 TCR-IgG1 | SJL x AKR | Anti-Vβ-8.1/2 | 0±0 (33) | 0.6±0.2 (33) | 0.1±0.1 (7) | 1.6±0.5 (7) |
| D10 TCR-IgG1 | AKR x SJL | Anti-D10 clonotype | 6.9±0.1 (5) | 4.6±0.2 (5) | ND | ND |

1) Data are presented as the man ± standard error of the mean (SEM). The total number of F1 animals investigated in each group is indicated in parentheses.
2) Splenic T cells were purified by immuno-affinity chromatography to yield lymphocyte preparations that contained 86-95% α/β TCR expressing cells and >3% B cells as determined by direct immunofluorescent staining of the cell surface.
3) Not done (ND) indicates that data has not yet been collected.

At 5-7 weeks of age the splenic T cells of (SJL x AKR)F1 mice derived from mice derived from unimmunized SJL mothers expressed the Vβ-8.2 and Vβ-8.3 members of the TCR Vβ-8 as expected. When the SJL mothers had been immunized with D10 TCR-IgG1, the F1 animals received maternal antibodies which could be shown by indirect immunofluorescence to recognize Vβ-8.2 family specific epitopes. Splenic T cells in these F1 mice failed to express TCR containing Vβ-8.2 segments and the expression of Vβ-8.3 segments was greatly reduced.

The removal of peripheral T cells utilizing Vβ-8 family genes appeared to result specifically from immunization of the SJL mothers with D10 TCR-IgG1, because in (SJL x AKR)F1 mice born of mothers immunized with BDC 2.5 TCR-IgG1 containing a Vβ-4 segment, the expression of Vβ-8 family genes occurred normally in the splenic T cell population. In further support of the specificity of maternal immunization in suppressing the Vβ-8 bearing peripheral T cell population, (AKR x SJL)F1 mice born of AKR mothers immunized with D10 TCR-IgG1 possessed D10 clonotype-specific antibodies, but at 5-7 weeks had a normal complement of splenic T cells bearing TCR containing Vβ-8.2 and Vβ-8.3 segments. It can be concluded therefore, that immunization of mothers against the antigens of a particular TCR Vβ-family results in the maternal transfer of specific antibodies that produce a long lasting depletion of T cells specifically utilizing gene members of the family.

Staphylococcal enterotoxin B (SEB) acts as a superantigen and *in vitro* activates all mature T cells utilizing Vβ-segments: 3, 7, 8.1/2/3, and 17 (Marrack, P. and J. Kappler, *Science 248*:705-711). The data described above indicates that maternal immunization with D10 TCR-IgG1 results in a long-lasting, and specific depletion of T cells expressing Vβ-8 genes in the offspring. The existence of Vβ-8 expressing cells at a level beneath the level of detection by indirect, cell-surface immunofluorescence yet in sufficient numbers to be activated by antigen remained a possibility. The presence of functional peripheral T cells with Vβ-8 TCR was tested by stimulating spleen cells with SEB *in vitro*. The cell population from each spleen was cultured separately for 4 days with SEB and then recombinant IL-2 was added twice, 2 days apart during a further 5 day incubation period. The total cell population from each culture was analyzed

individually by direct immunofluorescent staining of the cell surface with antibodies specific for Vβ-8.1/2, Vβ-8.3, TCR α/β and immunoglobulin. The data are presented in Table V.

Table V Maternal Immunization Against TCR Vβ-8 Family Epitopes Specifically Depletes the Peripheral T Cell Population of Vβ-8-Expressing T cells that Respond to the Superantigen SEB

| Soluble TCR immunization of mother | Mating: (female x male) | F1 anti-serum specificity | %[1] of SEB-activated spleen cells from 5-7 week old mice expressing[2]: | | | |
|---|---|---|---|---|---|---|
| | | | Vβ-8.2 TCR | Vβ-8.3 | TCR α/β | Ig |
| non-immunized | SJL x AKR | none | 13.8±0.7 (10) | 15.3±1.0 (10) | 99.6±0.2 (10) | 0.7±0.3 (10) |
| BDC 2.5 TCR-IgG1 | SJL x AKR | Anti-BDC 2.5 clonotype | 21.6±11.2 (5) | 21.8±1.8 (5) | 98.9±0.6 (5) | 0.3±0.2 (5) |
| D10 TCR-IgG1 | SJL x AKR | Anti-Vβ-8.1/2 | 0.6±0.2 (12) | 5.9±1.8 (12) | 98.8±0.4 (12) | 0.2±0.1 (12) |
| D10 TCR-IgG1 | AKR x SJL | Anti-D10 clonotype | 14.2±0.4 (5) | 14.6±0.3 (5) | 99.8±0 (5) | 0.2±0 (5) |

1) Data are presented as the mean ± standard error of the mean (SEM). The total number of F1 animals investigated in each group is indicated in parentheses.

2) Cells harvested from the cultures were analyzed by direct immunofluorescent staining of the cells surface.

*In vitro*, incubation of the spleen cells with SEB, and further stimulation with IL-2 resulted in lymphoid populations that were virtually all T cells as judged by immunofluorescent staining with MAb specific for the TCR β-chain. This was expected because SEB selectively stimulates the oligoclonal proliferation of T cells, and

IL-2 is mitogenic for the activated T cells. The data of Table V indicate that, regardless of whether the mothers were immunized, SEB triggered T cell expansion to a similar degree in spleen cell cultures of all the offspring. D10 TCR-IgG1 immunization of SJL mothers, however, resulted in a strong inhibition of expansion of T cells bearing TCR Vβ-8. Vβ-8.1/2-bearing T cells were virtually absent from the SEB stimulated populations, and the proportion of Vβ-8.3-bearing cells was reduced to less than 50% of that in cultures of spleen cells from (SJL x AKR)F1 mice derived from non-immunized SJL mothers. As with the effect of maternal immunization on the level of T cells bearing Vβ-8 in the spleens of F1 mice, the reduction in the proportion of Vβ-8-bearing T cells stimulated by SEB was immunologically specific. Immunization of SJL mothers with the BDC 2.5 TCR-IgG1 and AKR mothers with D10 TCR-IgG1 did not significantly affect the proportion of Vβ-8-bearing T cells stimulated by SEB in cultures of F1 spleen cells. It should be noted that the Vβ-8.2 segment of the D10 TCR shares family antigenic determinants with the Vβ-8.1 and 8.3 sub members. The data of the Table V indicate that D10 TCR-IgG1 maternal immunization has a greater inhibitory effect on the population of T cells bearing the Vβ-8.1/2 common epitope, recognized by the MAb MR5-2, than Vβ-8.3 bearing T cells. One interpretation of the data is that when SJL mice are immunized with D10 TCR-IgG1, the Vβ-8.2 epitopes stimulated a stronger antibody response than epitopes that are common to 8.1/2 and 3 Vβ-segments. Less profound suppression of the Vβ-8.3 bearing peripheral T cell population was also indicated by the presence of a small (2% approximately) but significant proportion of Vβ-8.3 bearing T cells in the spleens of (SJL x ARK)F1 mice born of D10 TCR-IgG1 immunized SJL mothers, whereas the level of cells bearing the Vβ-8.1/2 epitope was

statistically insignificant. The data are shown above in Table IV.

In conclusion, the data indicate that specific maternal immunization against a Vβ family can result in the transfer to the offspring of serum antibodies that deplete the peripheral lymphocyte population of T cells bearing Vβ segments of the family against which immunization is performed. In addition, the level of depletion is sufficient to diminish significantly the contribution of these T cells to a superantigen response. In autoimmune diseases where the pathology is to some extent due to oligoclonal activation of T cells utilizing a restricted set of Vβ gene family members, similar immunization strategies designed to stimulate an antibody response against the relevant family-specific epitopes could reduce the severity of disease by clonally depleting the pathogenic T cells.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: SOLUBLE HETERODIMERIC T CELL RECEPTORS

(iii) NUMBER OF SEQUENCES: 32

(iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: USA
(F) ZIP: 02173

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/367,589
(B) FILING DATE: January 3, 1995
(C) CLASSIFICATION:

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Carroll, Alice O.
(B) REGISTRATION NUMBER: 33,542
(C) REFERENCE/DOCKET NUMBER: PRO94-04A PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (617) 861-6240
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 459 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..456

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GAC AAG ATC CTG ACA GCA TCA TGT TTA CTC CTA GGC CTT CAC CTA          48
Met Asp Lys Ile Leu Thr Ala Ser Cys Leu Leu Leu Gly Leu His Leu
 1               5                  10                  15

GCT GGG GTG AGT GGC CAG CAG AAG GAG AAA AGT GAC CAG CAG CAG GTG          96
Ala Gly Val Ser Gly Gln Gln Lys Glu Lys Ser Asp Gln Gln Gln Val
            20                  25                  30

AGA CAA AGT CCC CAA TCT CTG ACA GTC TGG GAA GGA GAG ACC ACA ATT         144
Arg Gln Ser Pro Gln Ser Leu Thr Val Trp Glu Gly Glu Thr Thr Ile
        35                  40                  45

CTG AAC TGC AGT TAT GAG GAC AGC ACT TTT GAC TAC TTC CCA TGG TAC         192
Leu Asn Cys Ser Tyr Glu Asp Ser Thr Phe Asp Tyr Phe Pro Trp Tyr
    50                  55                  60

CGG CAG TTC CCT GGG AAA AGC CCT GCA CTC CTG ATA GCC ATA AGT TTG         240
Arg Gln Phe Pro Gly Lys Ser Pro Ala Leu Leu Ile Ala Ile Ser Leu
65                  70                  75                  80

GTG TCC AAT AAA AAG GAA GAT GGA CGA TTC ACA ATC TTC TTC AAT AAA         288
Val Ser Asn Lys Lys Glu Asp Gly Arg Phe Thr Ile Phe Phe Asn Lys
                85                  90                  95

AGG GAG AAA AAG CTC TCC TTG CAC ATC ACA GAC TCT CAG CCT GGA GAC         336
Arg Glu Lys Lys Leu Ser Leu His Ile Thr Asp Ser Gln Pro Gly Asp
            100                 105                 110

TCA GCC ACC TAC TTC TGT GCA GCA ACA GGT AGC TTC AAT AAG TTG ACC         384
Ser Ala Thr Tyr Phe Cys Ala Ala Thr Gly Ser Phe Asn Lys Leu Thr
        115                 120                 125

TTT GGA ACT GGG ACC AGA CTG GCT GTG TGC CCA TAC ATC CAG AAC CCA         432
Phe Gly Thr Gly Thr Arg Leu Ala Val Cys Pro Tyr Ile Gln Asn Pro
    130                 135                 140

GAA CCT TCA GAC GTC CCC TGT GAT TAA                                    459
Glu Pro Ser Asp Val Pro Cys Asp
145                 150
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 152 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 1               5                  10                  15

Ala Gly Val Ser Gly Gln Gln Lys Glu Lys Ser Asp Gln Gln Gln Val
            20                  25                  30

Arg Gln Ser Pro Gln Ser Leu Thr Val Trp Glu Gly Glu Thr Thr Ile
        35                  40                  45

Leu Asn Cys Ser Tyr Glu Asp Ser Thr Phe Asp Tyr Phe Pro Trp Tyr
    50                  55                  60

Arg Gln Phe Pro Gly Lys Ser Pro Ala Leu Leu Ile Ala Ile Ser Leu
65                  70                  75                  80

Val Ser Asn Lys Lys Glu Asp Gly Arg Phe Thr Ile Phe Phe Asn Lys
                85                  90                  95

Arg Glu Lys Lys Leu Ser Leu His Ile Thr Asp Ser Gln Pro Gly Asp
            100                 105                 110

Ser Ala Thr Tyr Phe Cys Ala Ala Thr Gly Ser Phe Asn Lys Leu Thr
        115                 120                 125

Phe Gly Thr Gly Thr Arg Leu Ala Val Cys Pro Tyr Ile Gln Asn Pro
    130                 135                 140

Glu Pro Ser Asp Val Pro Cys Asp
145                 150
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 155 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 90..152

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GTCACTGAGA AAGAACAACA TCCTGAGAGT TATAGCTGAC CTGCTAGTCA CCACAGTCTC      60

TTCTGGATTT TAATTTAATT GGGAAGAGC AAT GAA AAC ATA CGC TCC TAC ATT       113
                               Asn Glu Asn Ile Arg Ser Tyr Ile
                               155                 160

ATT CAT GTT TCT ATG GCT GCA GCT GGA TGG GAT GAG CCA AGG               155
Ile His Val Ser Met Ala Ala Ala Gly Trp Asp Glu Pro
            165                 170
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Glu Asn Ile Arg Ser Tyr Ile Ile His Val Ser Met Ala Ala Ala
1 5 10 15

Gly Trp Asp Glu Pro
20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCTCTAGAAG ATCTCCATGG ACAAGATCCT GACAGCATCA TGTTTACTC 49

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 87 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGAATTCAGA TCTGATGGAG CAGTCGTTGA TCCACGTGGT ACCAGGTCTG CTGATGAACA 60

GGGGACGTCT GAACTGGGGT AGGTGGC 87

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCTCTAGAAG ATCTCATGAG TAACACTGCC TTCCCTGACC CCGCC 45

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 104 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GGAATTCAGA TCTCCGTCTA GTCGTGATGA ACCACGAGGT ACCAGATCAG CAGACGAACA 60

GTCTGCTCGG CCCCAGGCCT CGGCCGAGAT GTTCTGTGTG ACAG 104

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCCCTGTGAT TAATGAGGTA C 21

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CTCATTAATC ACAGGGGACG T 21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCGAGGCC TGGGGCCGAG CAGACTGTGG GTGATAACCA TGGTAC 46

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CATGGTTATC ACCCACAGTC TGCTCGGCCC CAGGCCTC 38

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTGGTACCC AGGGGTAGTG GTTGTAAGCC TTGCATATGT ACAGTCCCAG AAGTA 55

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAAGATCTCA TTTACCAGGA GAGTGGGAGA GGCTCTTCTC 40

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCTCTAGAAG ATCTCCATGG GCTCCATTTT CCTCAGTT 38

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCTCTAGAAG ATCTGCATGC ATTCCTTACA TGTTTCACTA 40

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCTCAGCTGG TACACG 16

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTGCTTCTGA TGGCTCAAAC ACAG 24

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGCTTGTCT GGTTGCTCCA 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTGCTCAGGC AGTAGCTATA 20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CCTCTAGAAG ATCTCCATGG GCTCCATTTT CCTCAGTT 38

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCTCTAGAAG ATCTTCATGA AAACATACGC TCCTACATTA 40

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTGGTAAACA TCACCATCAC CATCACTCAC CCGGGAAGTA ATGACTCGAG 50

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATCCTCGAG TCATTACTTC CCGGGTGAGT GATGGTGATG GTGATGTTTA CCAGGAGA 58

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CTGGTAAAGC ATGGCGACAT CCGCAATTCG GGGGGTAATG ACTCGAG 47

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATCCTCGAG TCATTACCCC CCGAATTGCG GATGTCGCCA TGCTTTACCA GGAGA 55

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CCCCTGTCAT CACCATCACC ATCACTCACC CGGGAAGTAA TGAGGTACCT CGAG 54

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 62 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GATCCTCGAG GTACCTCATT ACTTCCCGGG TGAGTGATGG TGATGGTGAT GACAGGGGAC 60

GT 62

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCCCTGTGCA TGGCGACATC CGCAATTCGG GGGGTAATGA GGTACCTCGA G 51

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GATCCTCGAG GTACCTCATT ACCCCCCGAA TTGCGGATGT CGCCATGCAC AGGGGACGT 59

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 470 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

A TGACTAACAC TGCCTTCCCT GACCCCGCCT GGAACACCAC CCTGCTATCT 51

TGGGTTGCTC TCTTTCTCCT GGGAACAAAA CACATGGAGG CTGCAGTCAC CCAAAGCCCA 111

AGAAACAAGG TGGCAGTAAC AGGAGGAAAG GTGACATTGA GCTGTAATCA GACTAATAAC 171

CACAACAACA TGTACTGGTA TCGGCAGGAC ACGGGGCATG GGCTGAGGCT GATCCATTAT 231

TCATATGGTG CTGGCAGCAC TGAGAAAGGA GATATCCCTG ATGGATACAA GGCCTCCAGA 291

CCAAGCCAAG AGAACTTCTC CCTCATTCTG GAGTTGGCTA CCCCCTCTCA GACATCAGTG 351

TACTTCTGTG CCAGCGGGGG ACAGGGGCGG GCTGAGCAGT TCTTCGGACC AGGGACACGA 411

CTCACCGTCC TAGAGGATCC ATCTCGGCCG AGGCCTGGGG CCGAGCAGAC TGTGGGTGA 470

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 0 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

## CLAIMS

What is claimed is:

1. A soluble heterodimeric T cell receptor comprising an α subunit connected by disulfide bonding to a β subunit.

2. The soluble heterodimeric T cell receptor of Claim 1, wherein the heterodimeric T cell receptor has a conformation that is functionally indistinguishable from that appearing on the surface of T cells.

3. The soluble heterodimeric T cell receptor of Claim 2, wherein the α subunit is a chimeric polypeptide comprising an α segment and chimeric partner.

4. The soluble heterodimeric T cell receptor of Claim 3, wherein the chimeric partner is a ζ chain.

5. The soluble heterodimeric T cell receptor of Claim 3, wherein the chimeric partner is the constant region of an IgG1 molecule.

6. The soluble heterodimeric T cell receptor of Claim 2, wherein the β subunit is a chimeric polypeptide comprising an β segment and chimeric partner.

7. The soluble heterodimeric T cell receptor of Claim 6, wherein the chimeric partner is a ζ chain.

8. The soluble heterodimeric T cell receptor of Claim 6, wherein the chimeric partner is an IgG1 molecule.

9. The soluble heterodimeric T cell receptor of Claim 2, wherein the α and β subunits comprise α and β segments that are isolated from cells selected from the group consisting of: D10 T cells, B10 T cells, BDC 2.5 T cells, and BDC 6.9 T cells.

10. A soluble dual chain T cell receptor, comprising an α segment connected by a disulfide bonding to a β segment.

11. A DNA molecule, comprising a nucleic acid sequence encoding an α subunit of a heterodimeric T cell receptor and a nucleic acid sequence encoding a β subunit of a heterodimeric T cell receptor.

12. The DNA molecule of Claim 11, wherein the α subunit is a chimeric polypeptide comprising an α segment and chimeric partner.

13. The DNA molecule of Claim 12, wherein the chimeric partner is a ζ chain.

14. The DNA molecule of Claim 12, wherein the chimeric partner is an IgG1 molecule.

15. The DNA molecule of Claim 11, wherein the β subunit is a chimeric polypeptide comprising an β segment and chimeric partner.

16. The DNA molecule of Claim 15, wherein the chimeric partner is a ζ chain.

17. The DNA molecule of Claim 15, wherein the chimeric partner is an IgG1 molecule.

18. The DNA molecule of Claim 11, wherein the $\alpha$ and $\beta$ subunits comprise $\alpha$ and $\beta$ segments that are isolated from cells selected from the group consisting of: D10 T cells, B10 T cells, BDC 2.5 T cells, and BDC 6.9 T cells.

19. A transfer vector comprising a DNA molecule, the DNA molecule comprising a nucleic acid sequence encoding an $\alpha$ subunit of a heterodimeric T cell receptor and a nucleic acid sequence encoding a $\beta$ subunit of a heterodimeric T cell receptor.

20. An antibody to a soluble heterodimeric T cell receptor, the heterodimeric T cell receptor comprising an $\alpha$ subunit connected by disulfide bonding to a $\beta$ subunit.

21. The antibody of Claim 20, wherein the antibody is linked to an agent selected from the group consisting of: cytotoxic drugs, toxins, enzymes, and radioactive substances.

22. A method of depleting pathogenic T cells in a mammal, comprising administering to the mammal a therapeutically effective amount of an antibody to a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

23. A method of inhibiting the activation of pathogenic T cells in a mammal, comprising administering to the mammal a therapeutically effective amount of an antibody to a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

24. An assay for identifying agents that inhibit the interaction of T cell receptor with a complex formed between an Major Histocompatibility Complex/Human Leukocyte Antigen Complex (MHC/HLA) molecule and an antigenic peptide of interest, comprising the steps of:

a) incubating a sample of soluble heterodimeric T cell receptor with the MHC/HLA molecule and antigenic peptide of interest, to allow the soluble heterodimeric T cell receptor to interact with the MHC/HLA molecule and antigenic peptide;

b) incubating a sample of soluble heterodimeric T cell receptor with the MHC/HLA molecule and antigenic peptide of interest, and the agent to be tested, to allow the soluble heterodimeric T cell receptor to interact with the MHC/HLA molecule and antigenic peptide; and

c) evaluating the level of interaction between the soluble heterodimeric T cell receptor and the complexes formed between the MHC/HLA molecules and antigenic peptide in the presence of the agent to be tested and in the absence of the agent to be tested,

wherein less interaction between the soluble heterodimeric T cell receptor and the complexes formed between the MHC/HLA molecules and antigenic peptide in the presence of the agent to be tested than in the absence of the agent to be tested, is indicative that the agent inhibits the interaction between T cell receptor and the complexes formed between the MHC/HLA molecules and antigenic peptide.

25. An agent identified by the assay of Claim 24.

26. An assay for identifying agents that inhibit the interaction of T cell receptor with a T cell receptor specific antibody of interest, comprising the steps of:

a) incubating a sample of soluble heterodimeric T cell receptor with the T cell receptor specific antibody, to allow the soluble heterodimeric T cell receptor to interact with the T cell receptor antibody;

b) incubating a sample of soluble heterodimeric T cell receptor with the T cell receptor specific antibody and the agent to be tested, to allow the soluble heterodimeric T cell receptor to interact with the T cell receptor specific antibody; and

c) evaluating the level of interaction between the soluble heterodimeric T cell receptor and the T cell receptor specific antibody in the presence of the agent to be tested and in the absence of the agent to be tested,

wherein less interaction between the soluble heterodimeric T cell receptor and the T cell receptor specific antibody in the presence of the agent to be tested than in the absence of the agent to be tested, is indicative that the agent inhibits the interaction between T cell receptor and the T cell receptor specific antibody.

27. An agent identified by the assay of Claim 26.

28. An assay for detecting the presence of pathogenic T cells, comprising the steps of:

a) incubating a sample of lymphocytes with an antibody to a soluble heterodimeric T cell receptor that has a native-like conformation of T

cell receptors present on a pathogenic T cell, thereby generating a test sample; and

b) evaluating the test sample for the presence of interaction between the lymphocytes and the antibody,

wherein the presence of interaction between the lymphocytes and the antibody is indicative of the presence of pathogenic T cells.

29. A method of reducing the activation of pathogenic T cells in a mammal, comprising administering to the mammal a therapeutically effective amount of a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

30. A method of immunizing a mammal against T cell receptor antigenic structures on the surface of pathogenic T cells, comprising administering to the mammal an effective amount of a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the pathogenic T cells.

31. A method of depleting pathogenic T cells in the offspring of a female mammal, comprising administering to the mammal, during gestation or before weaning of the offspring, an amount of a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the pathogenic T cells, the amount being sufficient to generate an antibody response in the mammal.

32. A method of inhibiting the activation of pathogenic T cells in the offspring of a female mammal, comprising administering to the mammal, during gestation or before weaning of the offspring, an amount of a

soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the pathogenic T cells, the amount being sufficient to generate an antibody response in the mammal.

33. A method of depleting peripheral lymphocyte T cells which are involved in T cell mediated disease in a mammal, comprising administering to the mammal a therapeutically effective amount of an antibody to a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the peripheral lymphocyte T cells.

34. A method of depleting peripheral lymphocyte T cells which are involved in T cell mediated disease, in the offspring of a female mammal, comprising administering to the mammal, during gestation or before weaning of the offspring, an amount of a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the peripheral lymphocyte T cells, the amount being sufficient to generate an antibody response in the mammal.

35. A method of depleting peripheral lymphocyte T cells bearing the V$\beta$ segment of a family of T cells which are involved in a T cell mediated disease, in a mammal, comprising administering to the mammal a therapeutically effective amount of an antibody to a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the peripheral lymphocyte T cells.

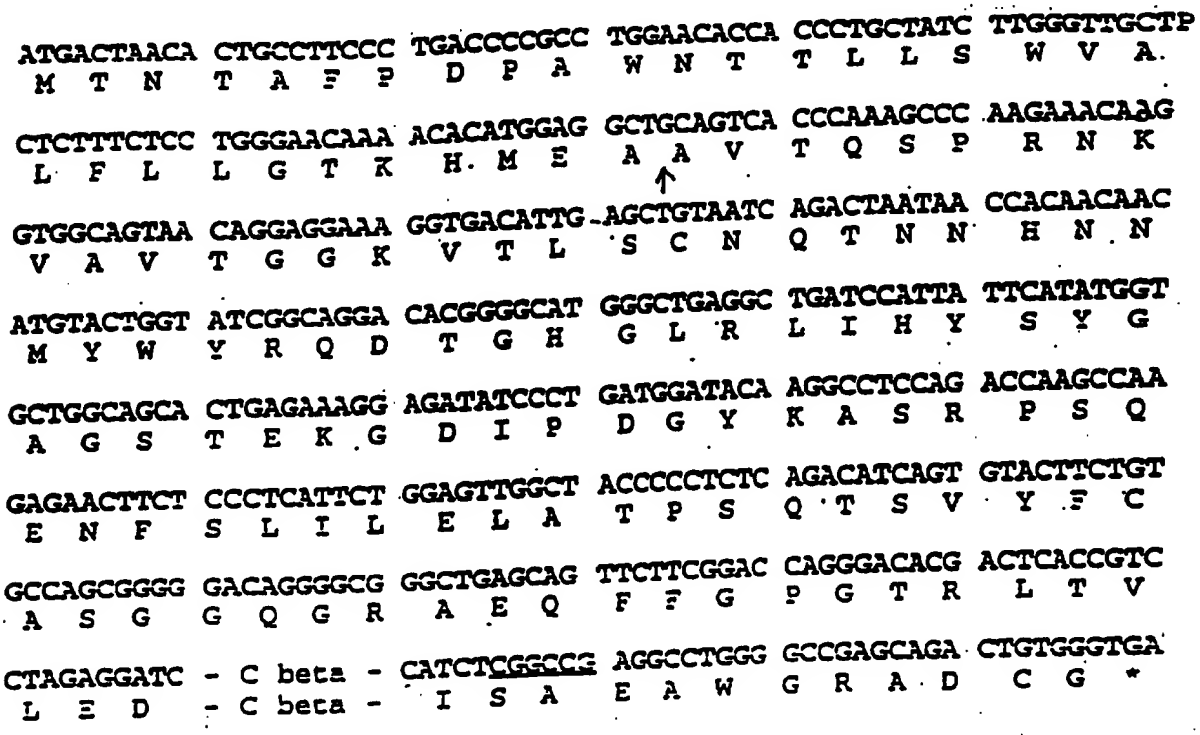
36. The method of Claim 35, wherein the T cell mediated disease is diabetes.

37. A method of depleting peripheral lymphocyte T cells bearing the Vβ segment of a family of T cells which are involved in a T cell mediated disease, in the offspring of a female mammal, comprising administering to the mammal, during gestation or before weaning of the offspring, an amount of a soluble heterodimeric T cell receptor that has a native-like conformation of receptors on the peripheral lymphocyte T cells, the amount being sufficient to generate an antibody response in a mammal.

38. The method of Claim 37 wherein the T cell mediated disease is diabetes.

```
ATGGACAAGA TCCTGACAGC ATCATGTTTA CTCCTAGGCC TTCACCTAGC TGGGGTGAGT
 M  D  K   I  L  T  A   S  C  L   L  L  G   L  H  L  A   G  V  S

GGCCAGCAGA AGGAGAAAAG TGACCAGCAG CAGGTGAGAC AAAGTCCCCA ATCTCTGACA
 G  Q  Q   K  E  K  S   D  Q  Q   Q  V  R   Q  S  P  Q   S  L  T
                        ↑
GTCTGGGAAG GAGAGACCAC AATTCTGAAC TGCAGTTATG AGGACAGCAC TTTTGACTAC
 V  W  E   G  E  T  T   I  L  N   C  S  Y   E  D  S  T   F  D  Y

TTCCCATGGT ACCGGCAGTT CCCTGGGAAA AGCCCTGCAC TCCTGATAGC CATAAGTTTG
 F  P  W   Y  R  Q  F   P  G  K   S  P  A   L  L  I  A   I  S  L

GTGTCCAATA AAAAGGAAGA TGGACGATTC ACAATCTTCT TCAATAAAAG GGAGAAAAAG
 V  S  N   K  K  E  D   G  R  F   T  I  F   F  N  K  R   E  K  K

CTCTCCTTGC ACATCACAGA CTCTCAGCCT GGAGACTCAG CCACCTACTT CTGTGCAGCA
 L  S  L   H  I  T  D   S  Q  P   G  D  S   A  T  Y  F   C  A  A

ACAGGTAGCT TCAATAAGTT GACCTTTGGA ACTGGGACCA GACTGGCTGT GTGCCCATAC
 T  G  S   F  N  K  L   T  F  G   T  G  T   R  L  A  V   C  P  Y

ATCCAGAACC CAGAACCT - C alpha -  TCAGACGTCC CCTGTGATTA A
 I  Q  N   P  E  P  - C alpha -   S  D  V   P  C  D  *
```

# FIGURE 1


ATGACTAACA CTGCCTTCCC TGACCCCGCC TGGAACACCA CCCTGCTATC TTGGGTTGCTP
M T N T A F P D P A W N T T L L S W V A
CTCTTTCTCC TGGGAACAAA ACACATGGAG GCTGCAGTCA CCCAAAGCCC AAGAAACAAG
L F L L G T K H M E A A V T Q S P R N K
GTGGCAGTAA CAGGAGGAAA GGTGACATTG AGCTGTAATC AGACTAATAA CCACAACAAC
V A V T G G K V T L S C N Q T N N H N N
ATGTACTGGT ATCGGCAGGA CACGGGGCAT GGGCTGAGGC TGATCCATTA TTCATATGGT
M Y W Y R Q D T G H G L R L I H Y S Y G
GCTGGCAGCA CTGAGAAAGG AGATATCCCT GATGGATACA AGGCCTCCAG ACCAAGCCAA
A G S T E K G D I P D G Y K A S R P S Q
GAGAACTTCT CCCTCATTCT GGAGTTGGCT ACCCCCTCTC AGACATCAGT GTACTTCTGT
E N F S L I L E L A T P S Q T S V Y F C
GCCAGCGGGG GACAGGGGCG GGCTGAGCAG TTCTTCGGAC CAGGGACACG ACTCACCGTC
A S G G Q G R A E Q F F G P G T R L T V
CTAGAGGATC - C beta - CATCTCGGCCG AGGCCTGGG GCCGAGCAGA CTGTGGGTGA
L E D - C beta - I S A E A W G R A D C G *

# FIGURE 2

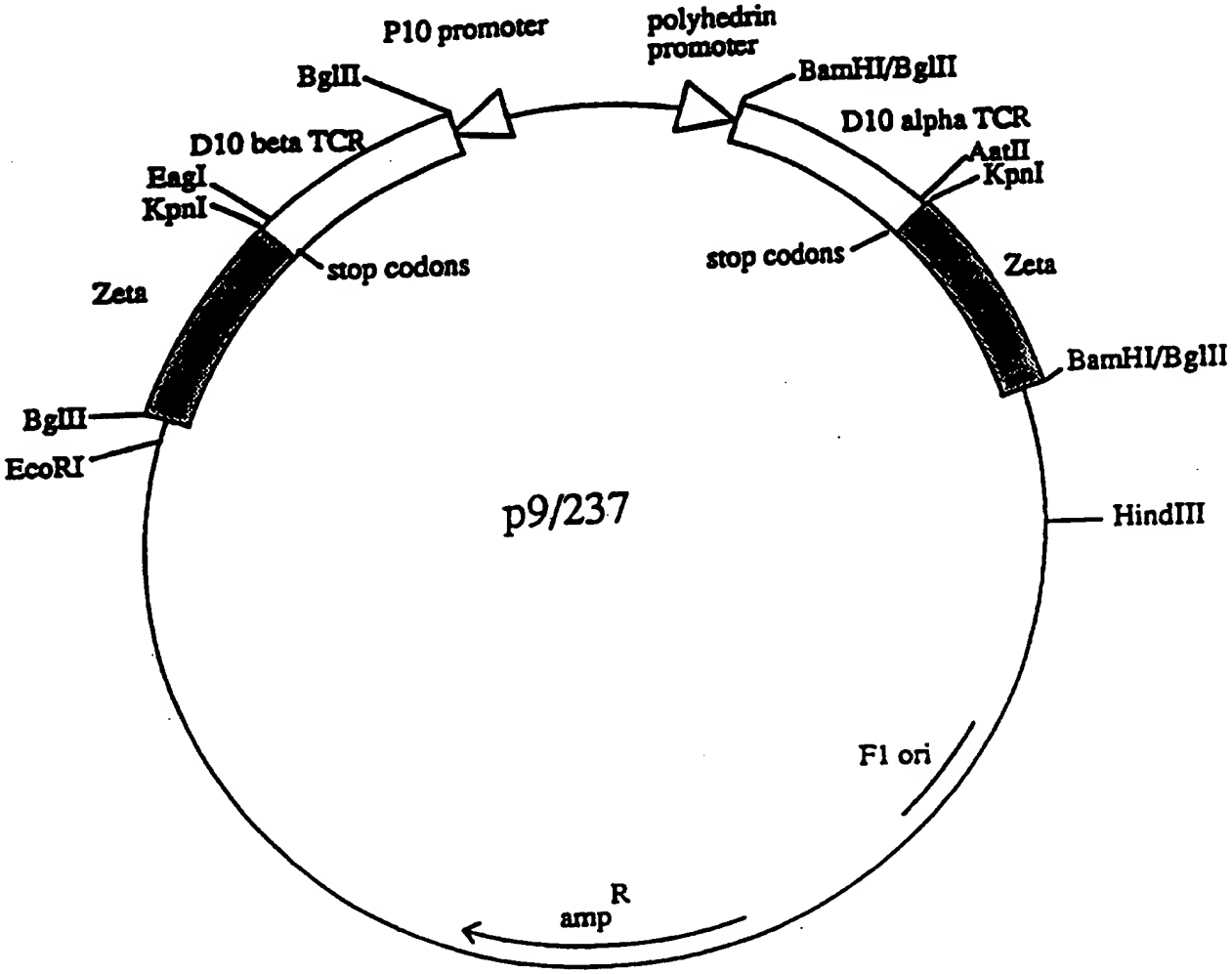
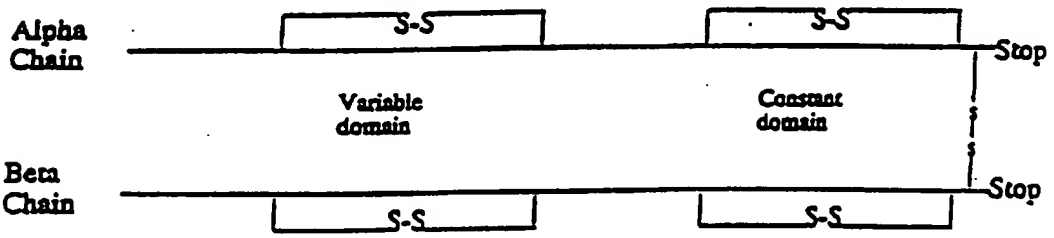

P10 promoter
polyhedrin promoter
BglII
BamHI/BglII
D10 beta TCR
D10 alpha TCR
AatII
EagI
KpnI
KpnI
stop codons
stop codons
Zeta
Zeta
BamHI/BglII
BglII
EcoRI
p9/237
HindIII
F1 ori
R
amp

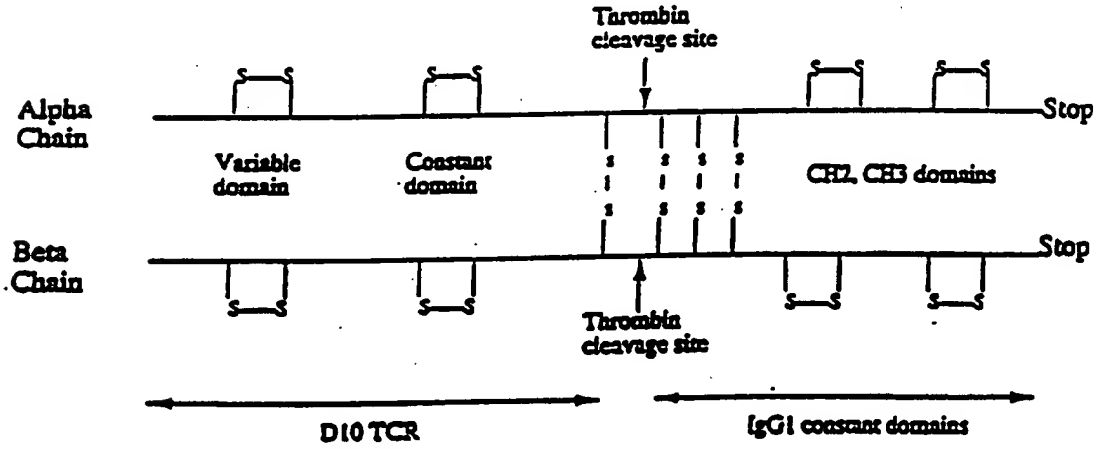
**FIGURE 3**

# FIGURE 4A

D10 TCR




D10-IgG1 TCR




# FIGURE 4B

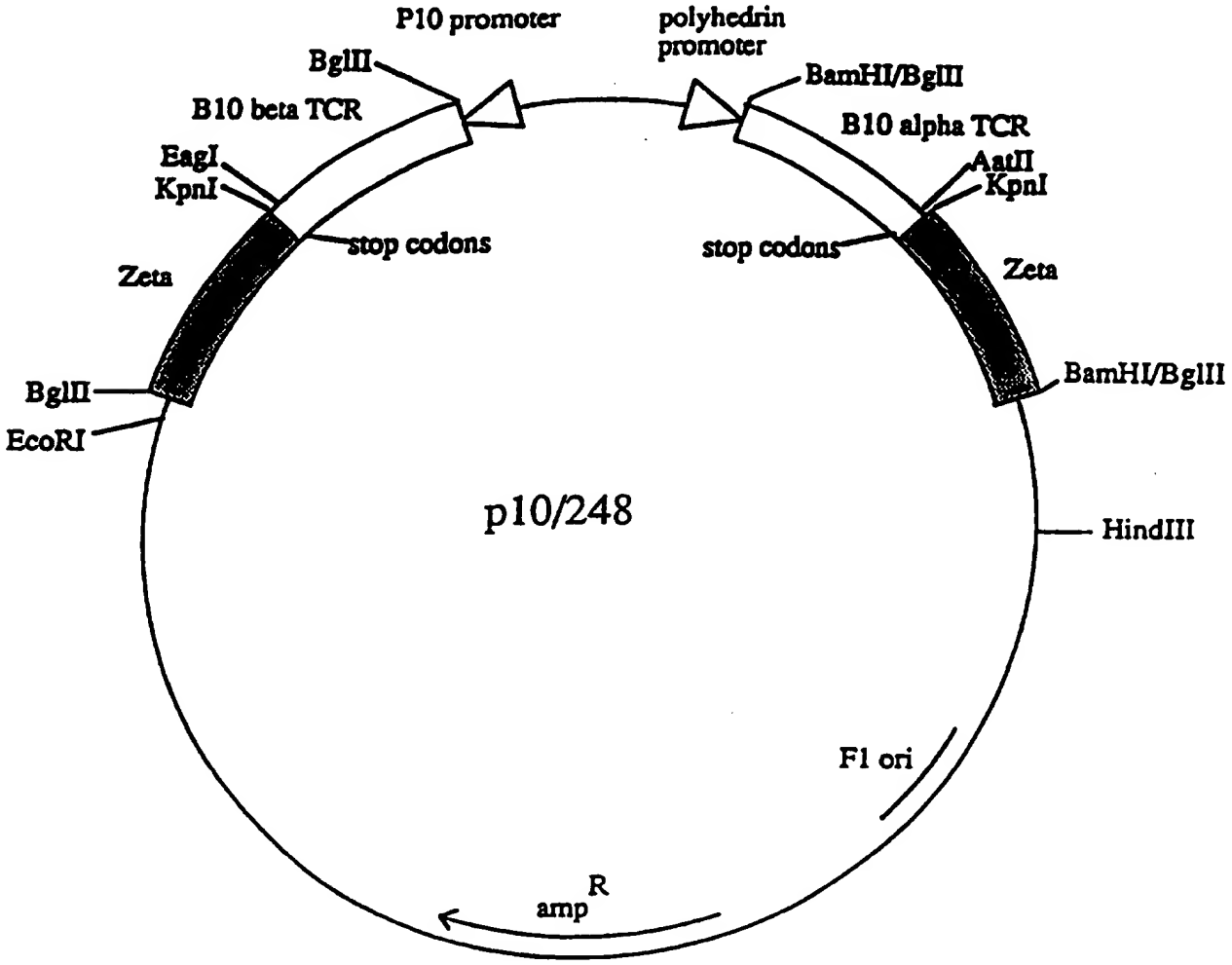

P10 promoter
polyhedrin promoter
BglII
BamHI/BglII
B10 beta TCR
B10 alpha TCR
EagI
KpnI
AatII
KpnI
stop codons
stop codons
Zeta
Zeta
BglII
BamHI/BglII
EcoRI
p10/248
HindIII
F1 ori
amp R

**FIGURE 5**

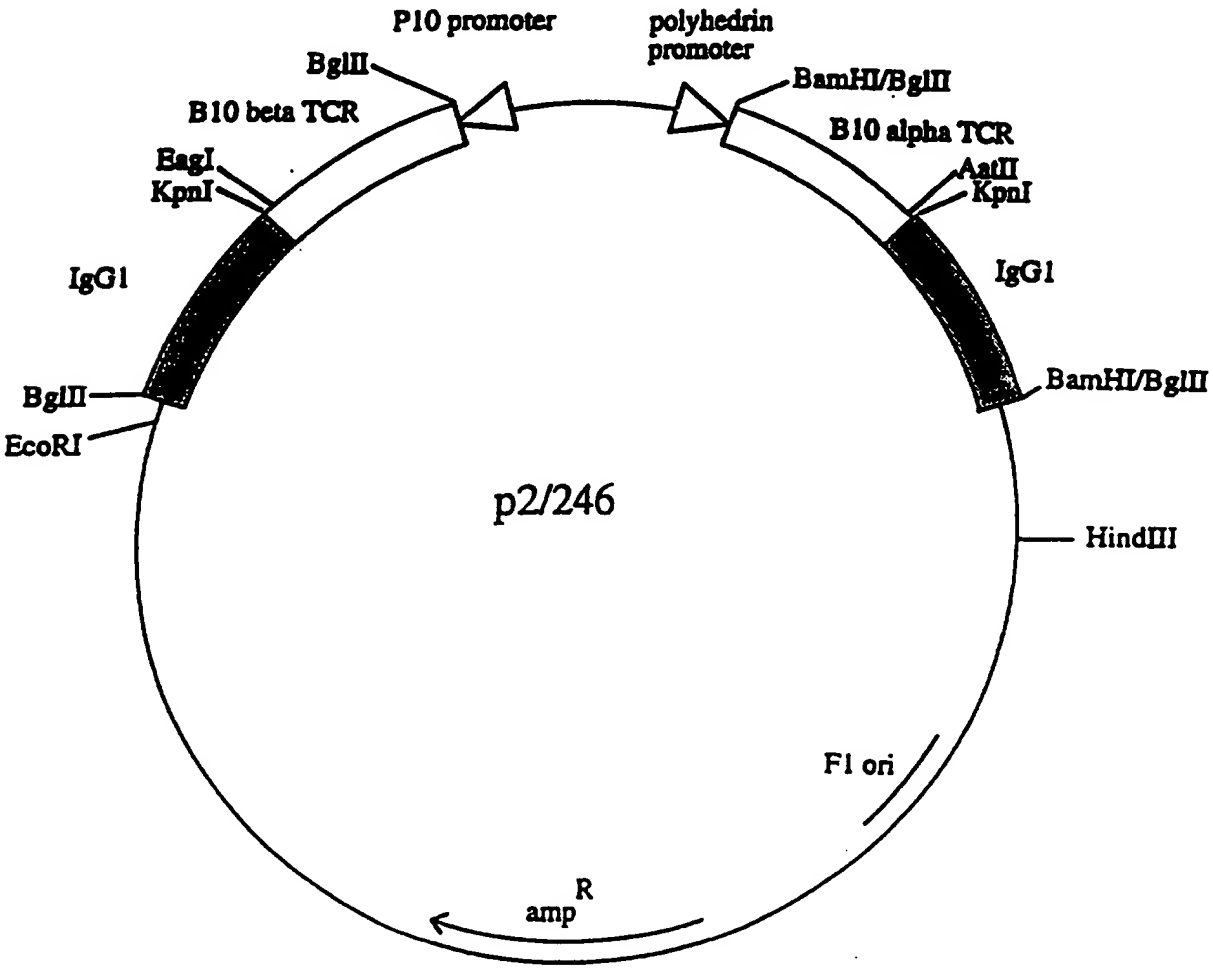
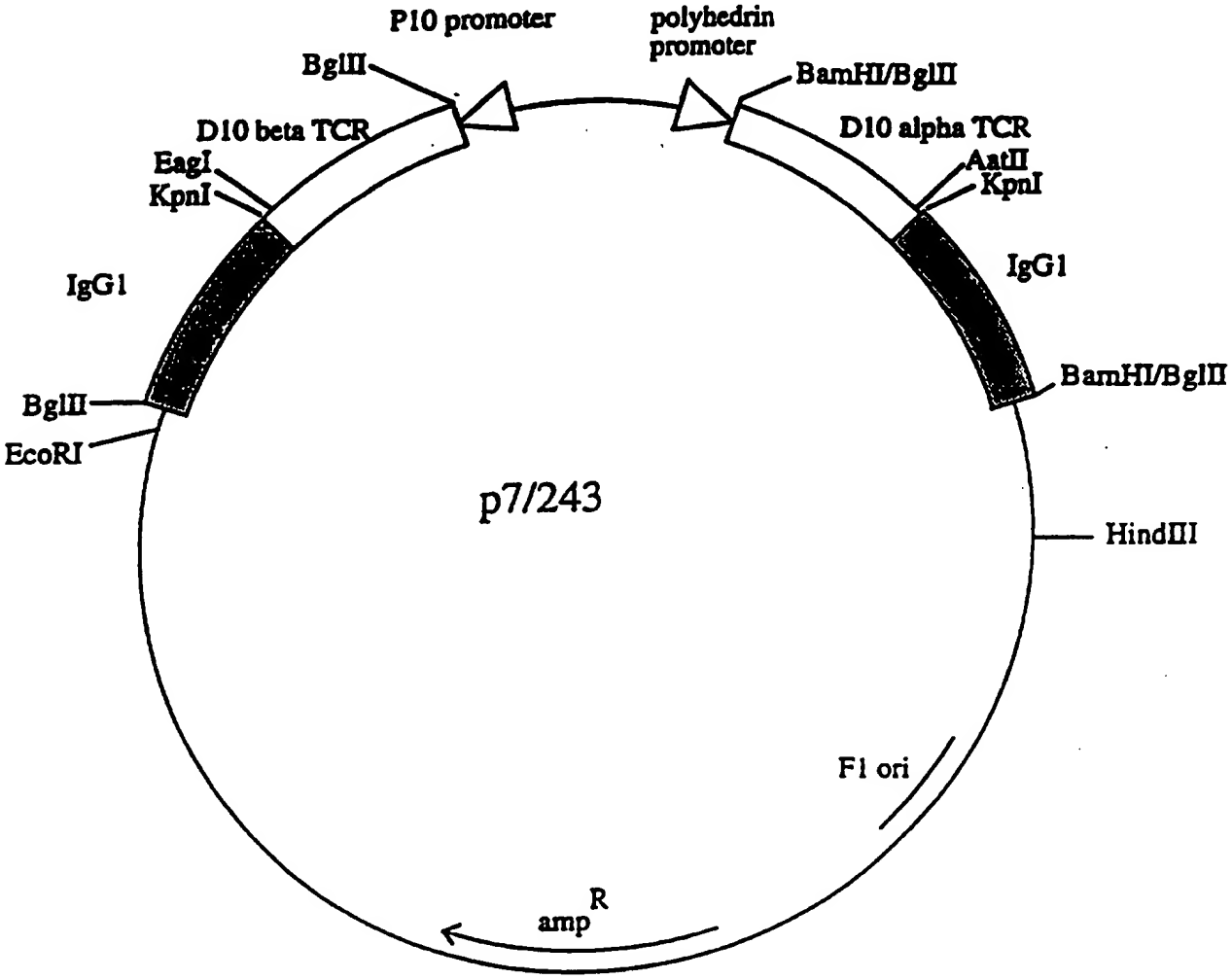

P10 promoter
polyhedrin promoter
BglII
BamHI/BglII
B10 beta TCR
B10 alpha TCR
EagI
KpnI
AatII
KpnI
IgG1
IgG1
BglII
EcoRI
BamHI/BglII
p2/246
HindIII
F1 ori
R
amp

**FIGURE 6**


P10 promoter
polyhedrin promoter
BglII
BamHI/BglII
D10 beta TCR
D10 alpha TCR
EagI
KpnI
AatII
KpnI
IgG1
IgG1
BglII
BamHI/BglII
EcoRI
p7/243
HindIII
F1 ori
amp R

# FIGURE 7

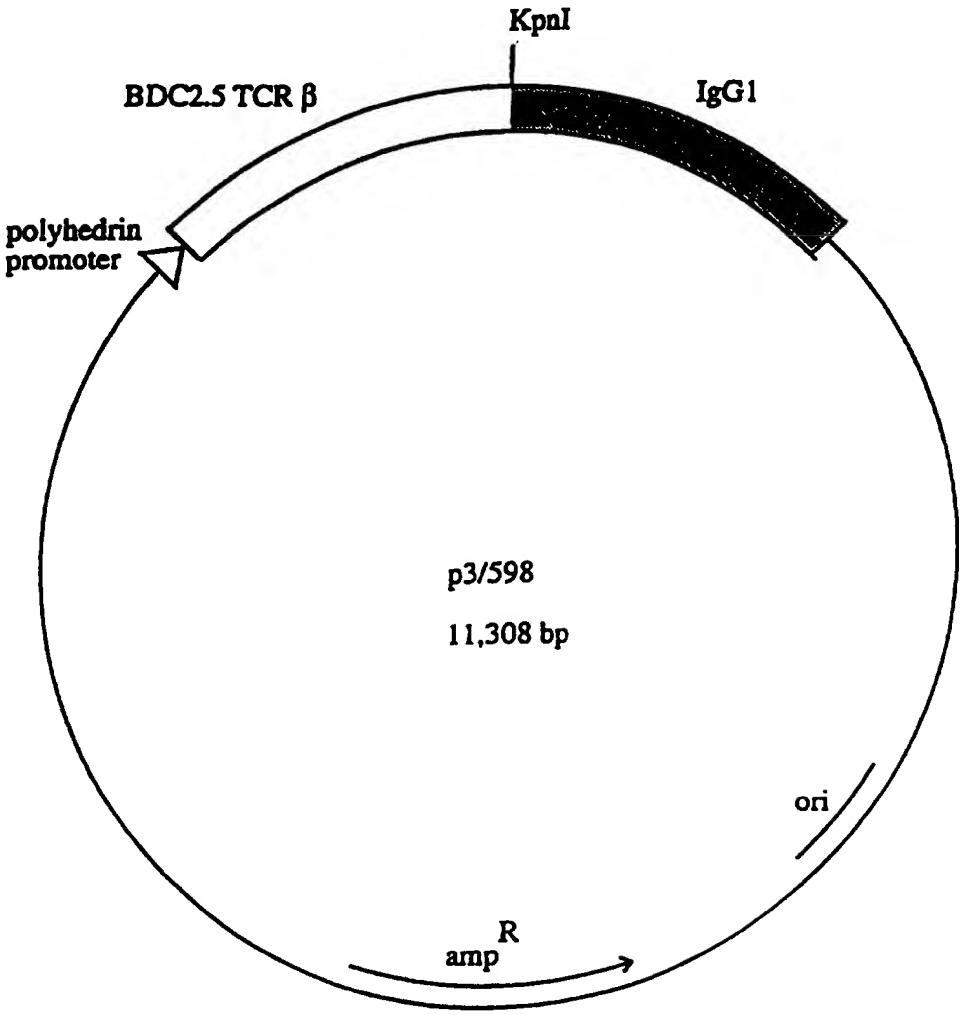

KpnI
BDC2.5 TCR β
IgG1
polyhedrin
promoter
p3/598
11,308 bp
ori
R
amp

**FIGURE 8**

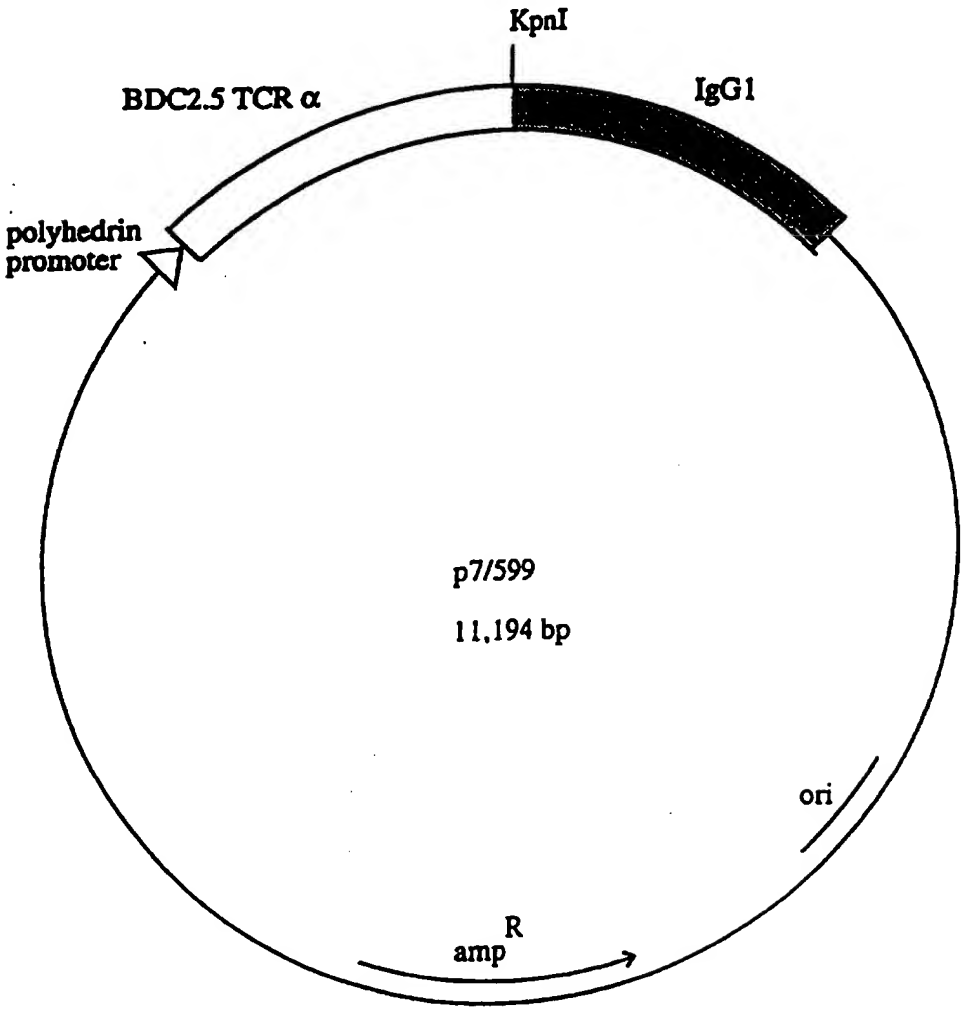

KpnI
BDC2.5 TCR α
IgG1
polyhedrin
promoter
p7/599
11,194 bp
ori
R
amp

**FIGURE 9**

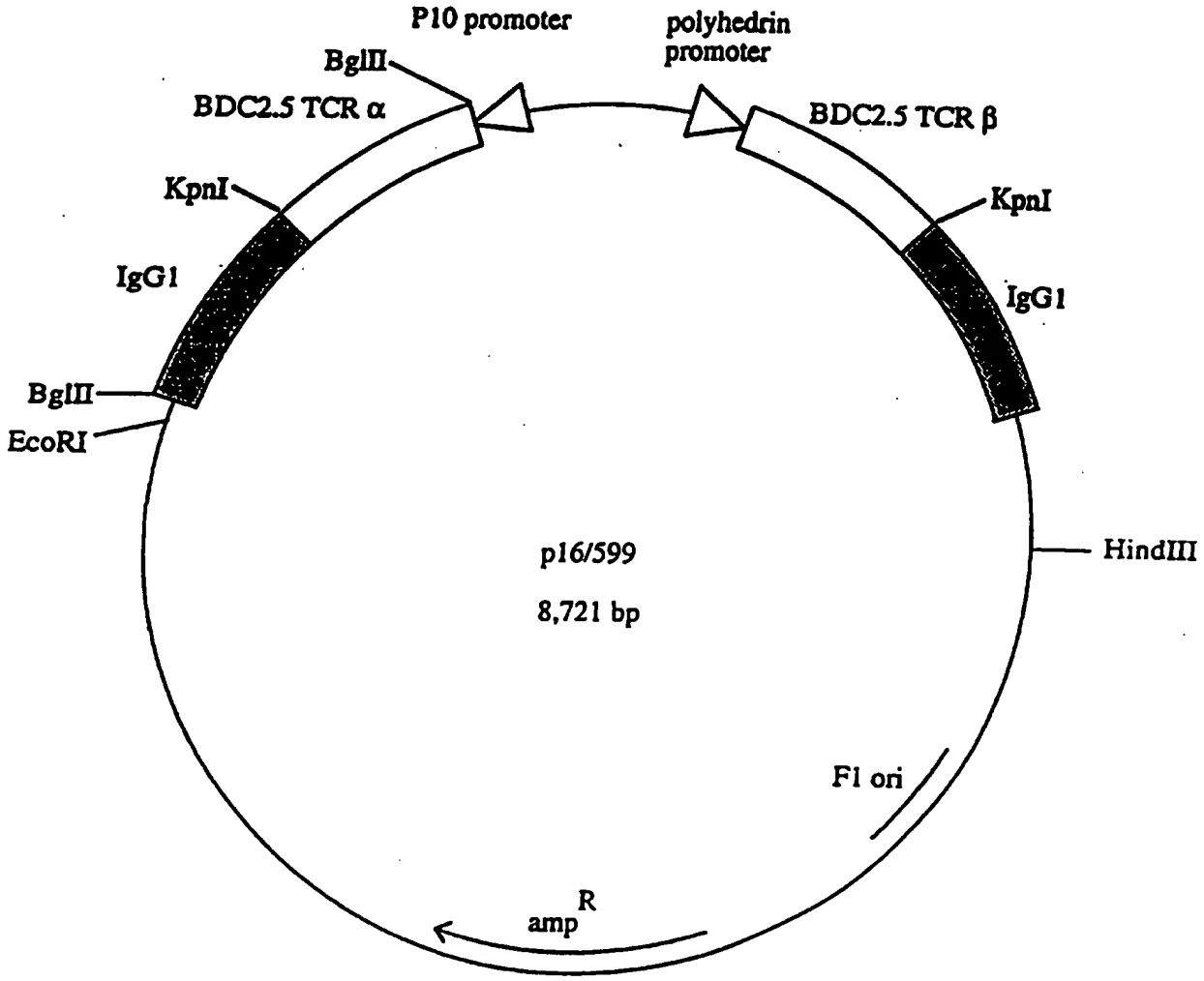

P10 promoter
polyhedrin promoter
BglII
BDC2.5 TCR α
BDC2.5 TCR β
KpnI
KpnI
IgG1
IgG1
BglII
EcoRI
p16/599
8,721 bp
HindIII
F1 ori
R
amp

**FIGURE 10**

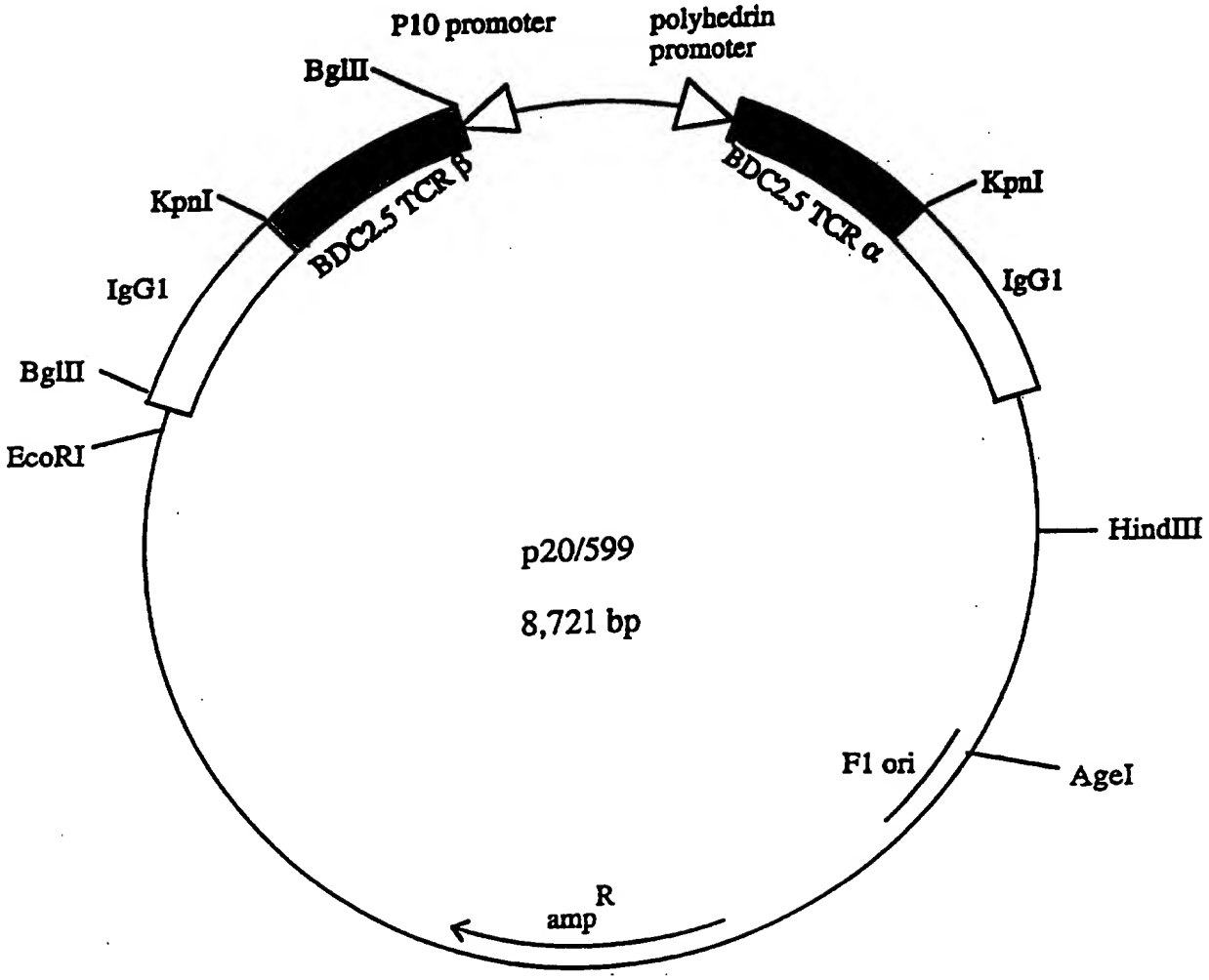
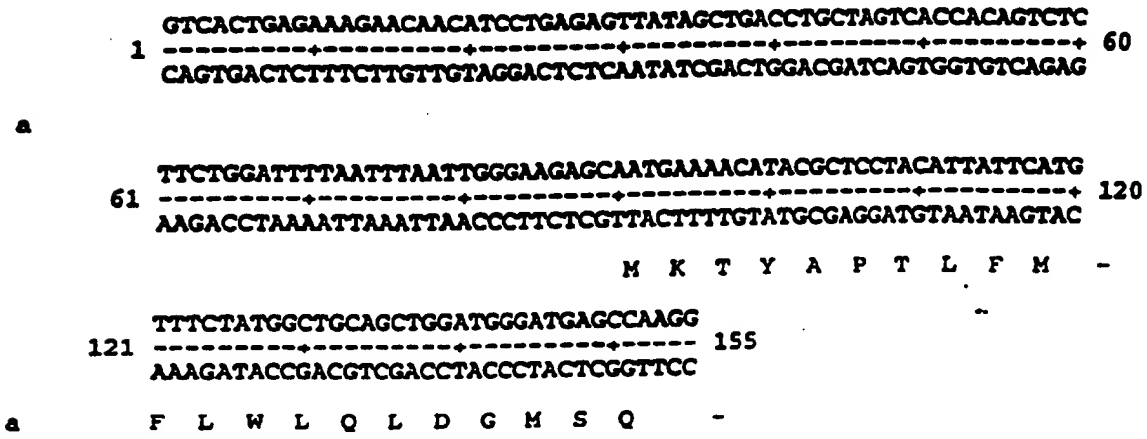

P10 promoter
polyhedrin promoter
BglII
KpnI
BDC2.5 TCR β
BDC2.5 TCR α
KpnI
IgG1
IgG1
BglII
EcoRI
HindIII
p20/599
8,721 bp
F1 ori
AgeI
R
amp

FIGURE 11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1
60
CAGTGACTCTTTCTTGTTGTAGGACTCTCAATATCGACTGGACGATCAGTGGTGTCAGAG
a
TTCTGGATTTTAATTTAATTGGGAAGAGCAATGAAAACATACGCTCCTACATTATTCATG
61
120
AAGACCTAAAATTAAATTAACCCTTCTCGTTACTTTTGTATGCGAGGATGTAATAAGTAC
M K T Y A P T L F M -
TTTCTATGGCTGCAGCTGGATGGGATGAGCCAAGG
121
155
AAAGATACCGACGTCGACCTACCCTACTCGGTTCC
a
F L W L Q L D G M S Q -

# FIGURE 12

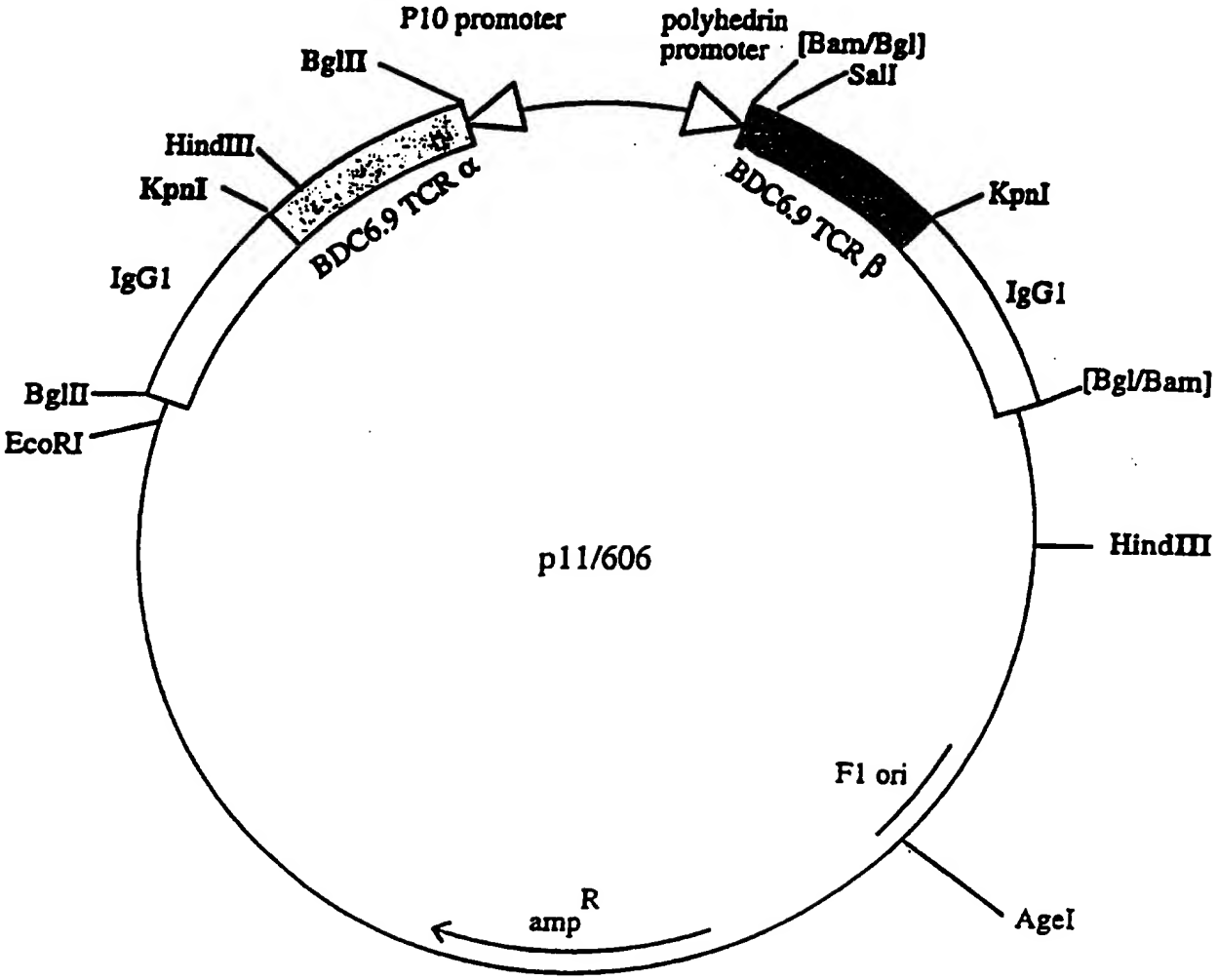
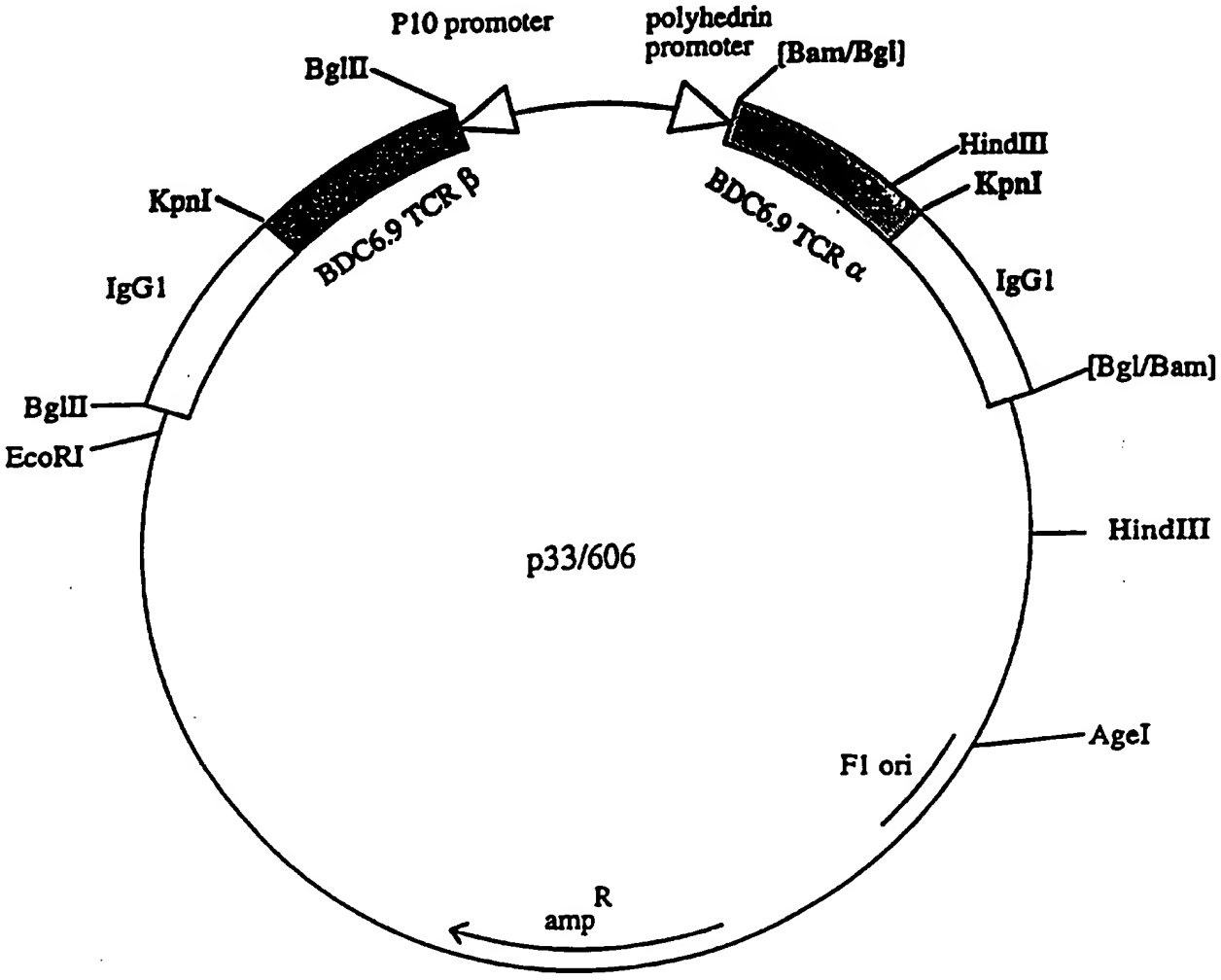

P10 promoter
polyhedrin promoter
[Bam/Bgl]
SalI
BglII
HindIII
KpnI
BDC6.9 TCR α
BDC6.9 TCR β
KpnI
IgG1
IgG1
BglII
EcoRI
[Bgl/Bam]
p11/606
HindIII
F1 ori
AgeI
amp R

**FIGURE 13**


P10 promoter
polyhedrin promoter
BglII
[Bam/Bgl]
HindIII
KpnI
KpnI
BDC6.9 TCR β
BDC6.9 TCR α
IgG1
IgG1
[Bgl/Bam]
BglII
EcoRI
HindIII
p33/606
AgeI
F1 ori
R
amp

# FIGURE 14

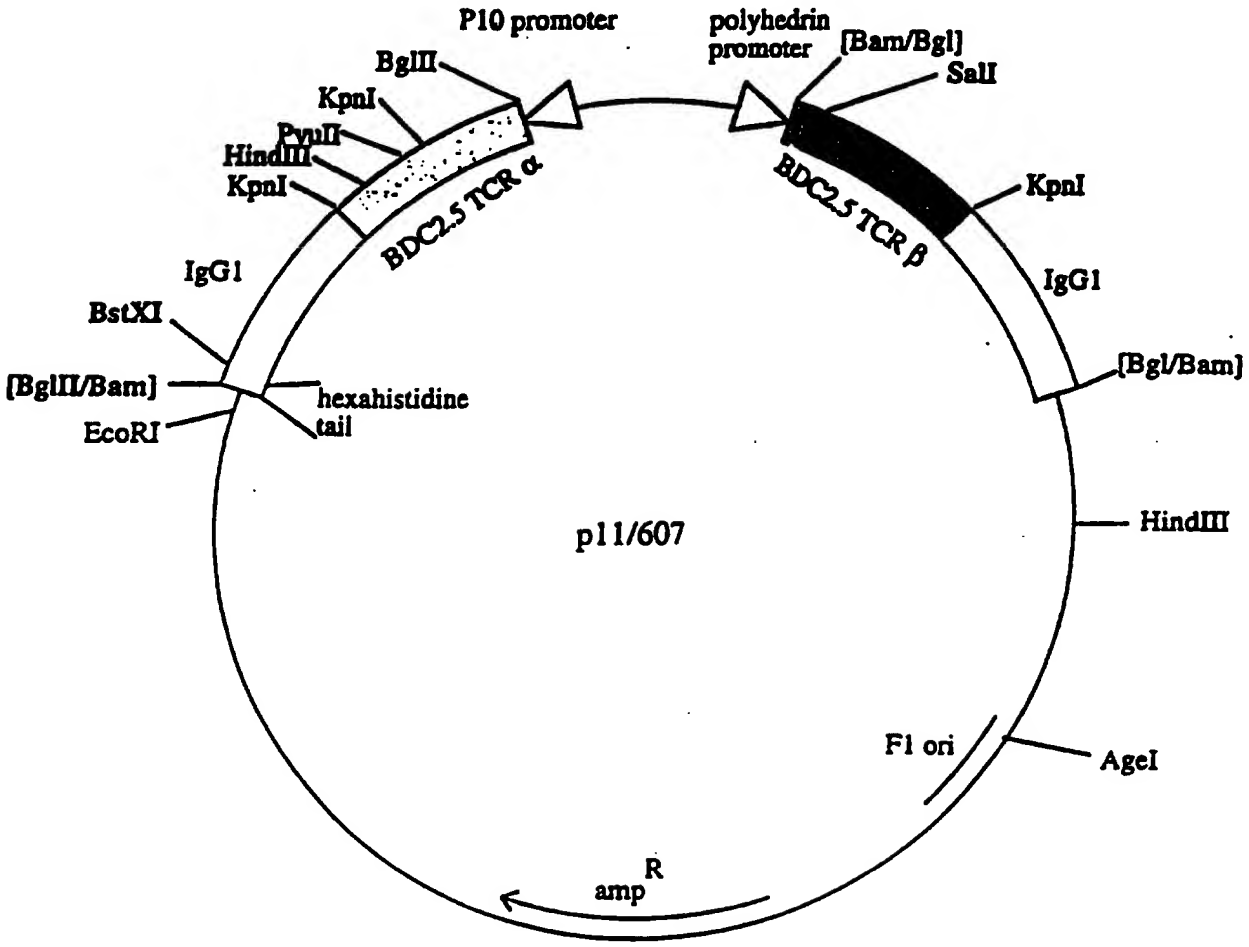
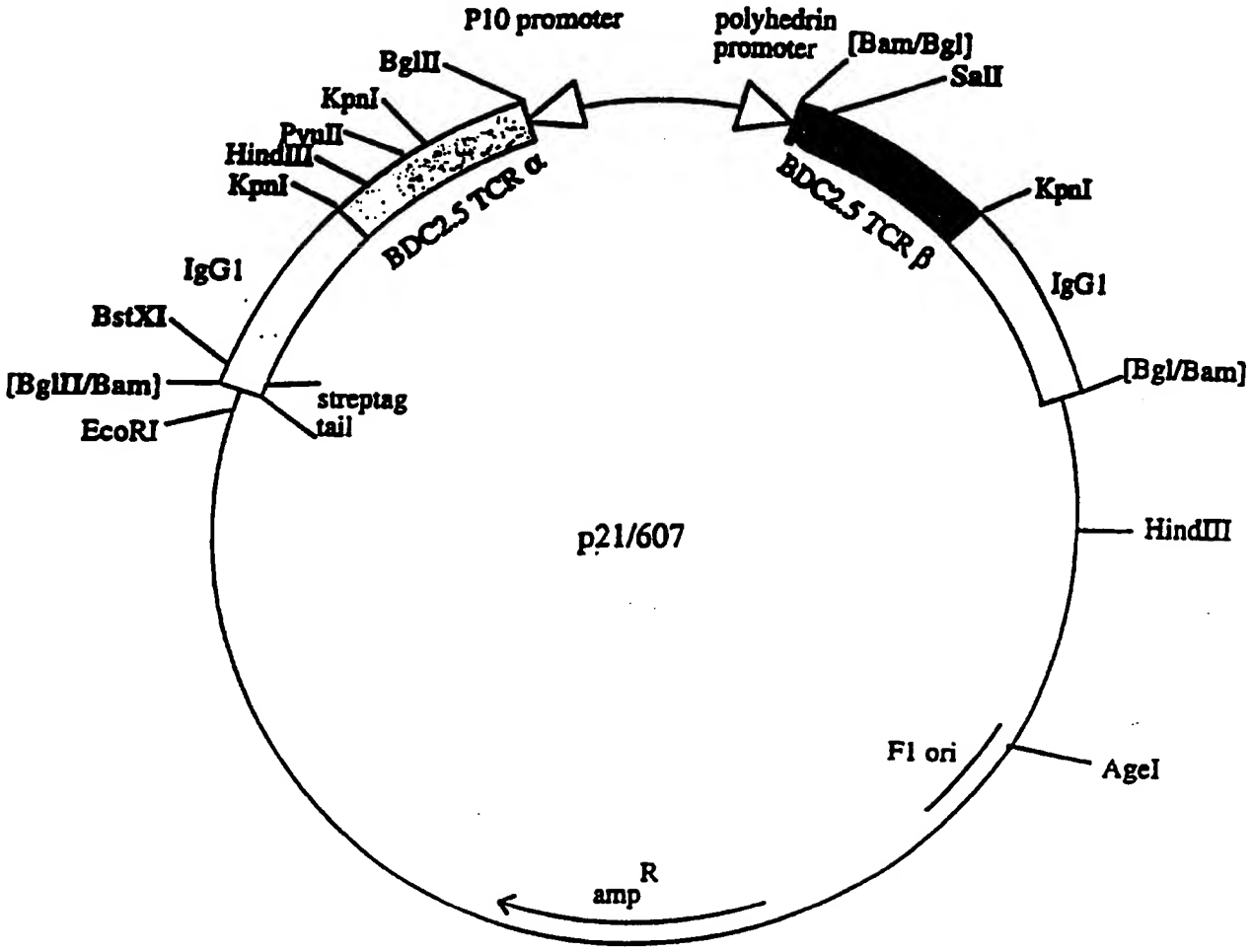

P10 promoter
polyhedrin promoter
[Bam/Bgl]
SalI
BglII
KpnI
PvuII
HindIII
KpnI
BDC2.5 TCR α
BDC2.5 TCR β
KpnI
IgG1
IgG1
BstXI
[BglII/Bam]
EcoRI
hexahistidine tail
[Bgl/Bam]
p11/607
HindIII
F1 ori
AgeI
R
amp

**FIGURE 15**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polyhedrin promoter
[Bam/Bgl]
SalI
BglII
KpnI
PvuII
HindIII
KpnI
BDC2.5 TCR α
BDC2.5 TCR β
KpnI
IgG1
IgG1
BstXI
[BglII/Bam]
EcoRI
streptag tail
[Bgl/Bam]
p21/607
HindIII
F1 ori
AgeI
R
amp

**FIGURE 16**

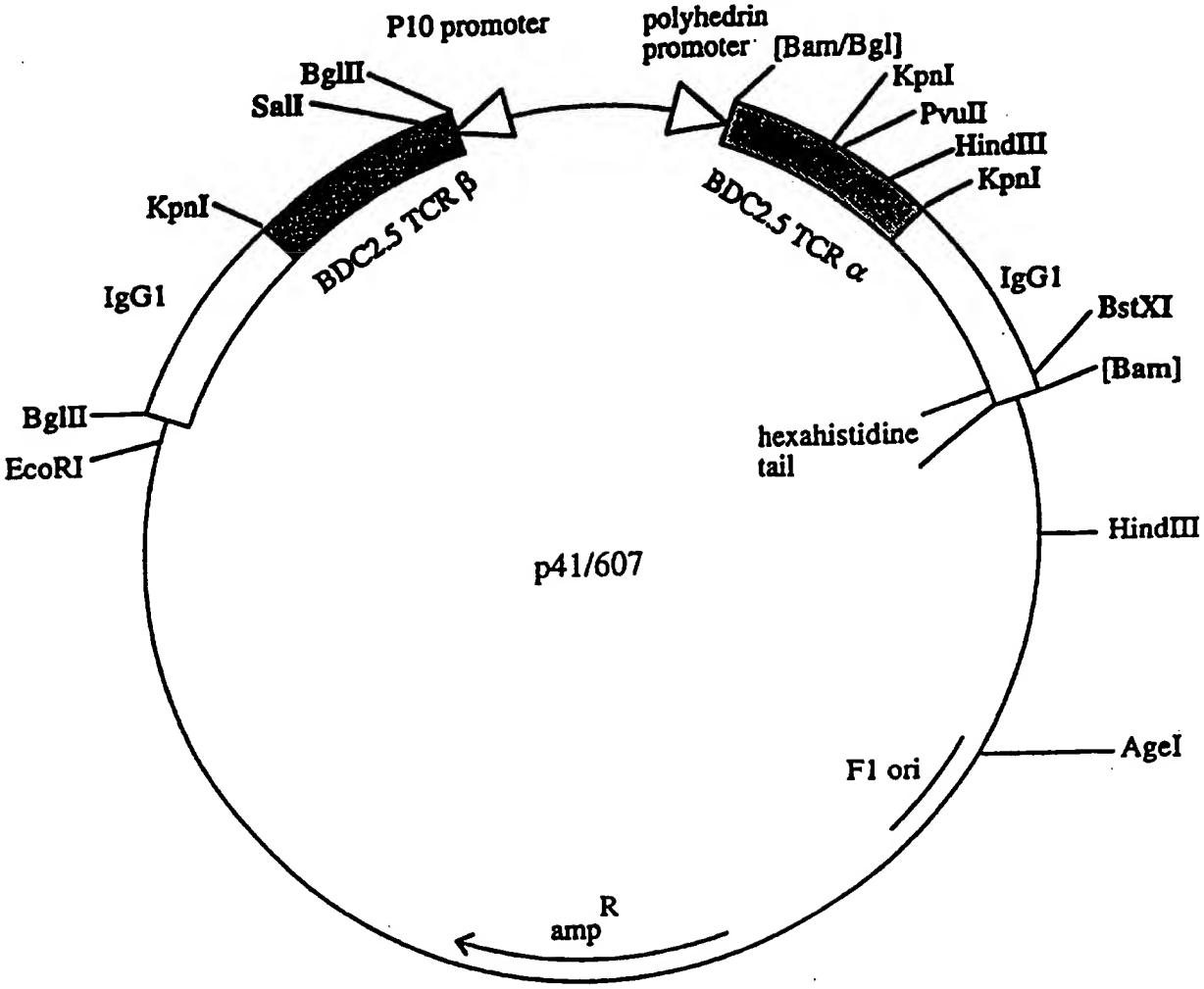

P10 promoter
polyhedrin promoter
[Bam/Bgl]
BglII
SalI
KpnI
PvuII
HindIII
KpnI
BDC2.5 TCR β
BDC2.5 TCR α
IgG1
IgG1
BstXI
[Bam]
BglII
EcoRI
hexahistidine tail
HindIII
p41/607
AgeI
F1 ori
amp R

**FIGURE 17**

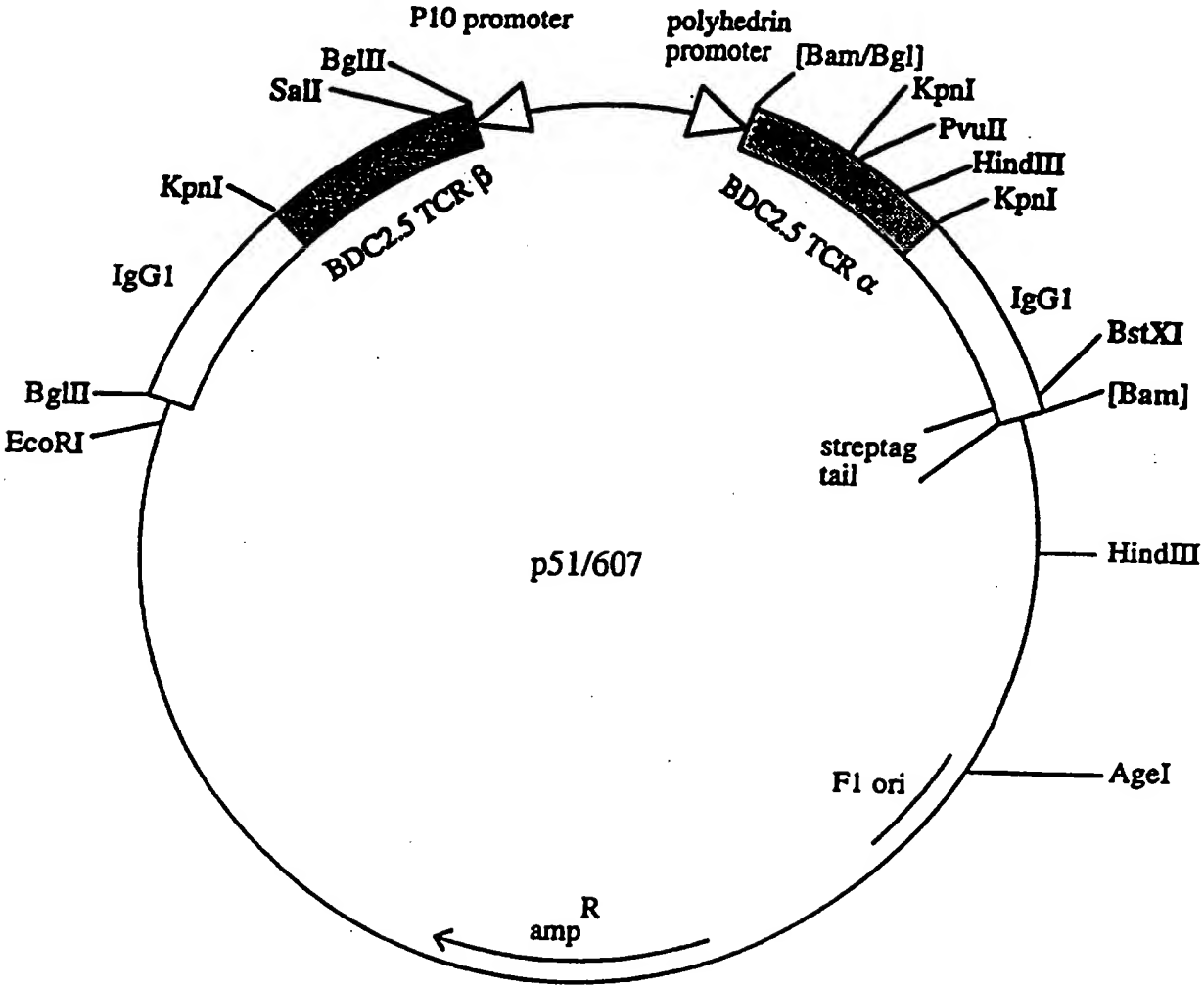

P10 promoter
polyhedrin promoter
[Bam/Bgl]
BglII
SalI
KpnI
PvuII
HindIII
KpnI
KpnI
BDC2.5 TCR β
BDC2.5 TCR α
IgG1
IgG1
BstXI
[Bam]
BglII
EcoRI
streptag tail
p51/607
HindIII
F1 ori
AgeI
amp R

**FIGURE 18**

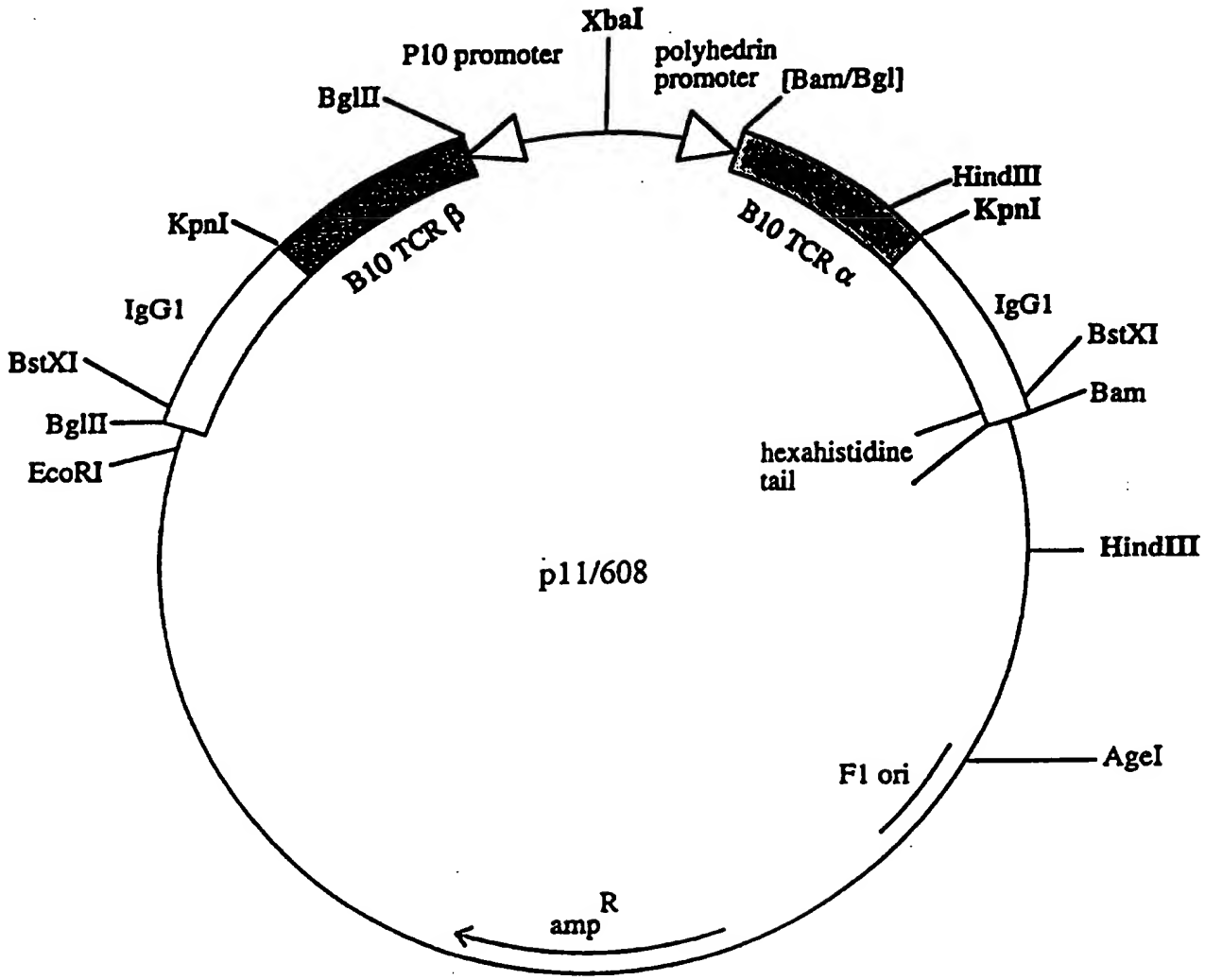
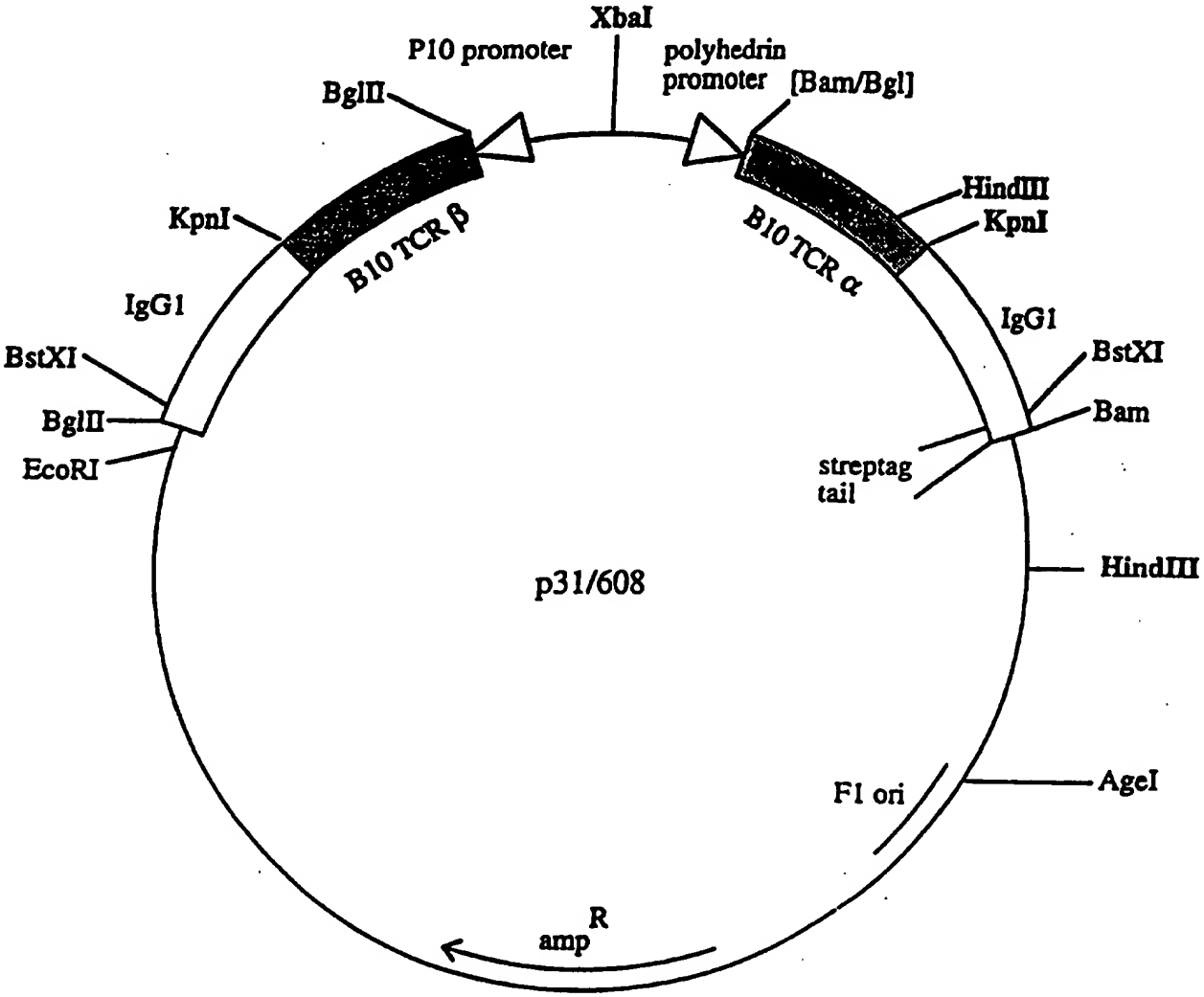

XbaI
P10 promoter
polyhedrin promoter
[Bam/Bgl]
BglII
HindIII
KpnI
KpnI
B10 TCR β
B10 TCR α
IgG1
IgG1
BstXI
BstXI
Bam
BglII
hexahistidine tail
EcoRI
HindIII
p11/608
AgeI
F1 ori
amp R

**FIGURE 19**


XbaI
P10 promoter
polyhedrin promoter
[Bam/Bgl]
BglII
HindIII
KpnI
KpnI
B10 TCR β
B10 TCR α
IgG1
IgG1
BstXI
BstXI
BglII
Bam
EcoRI
streptag tail
p31/608
HindIII
AgeI
F1 ori
R
amp

# FIGURE 20

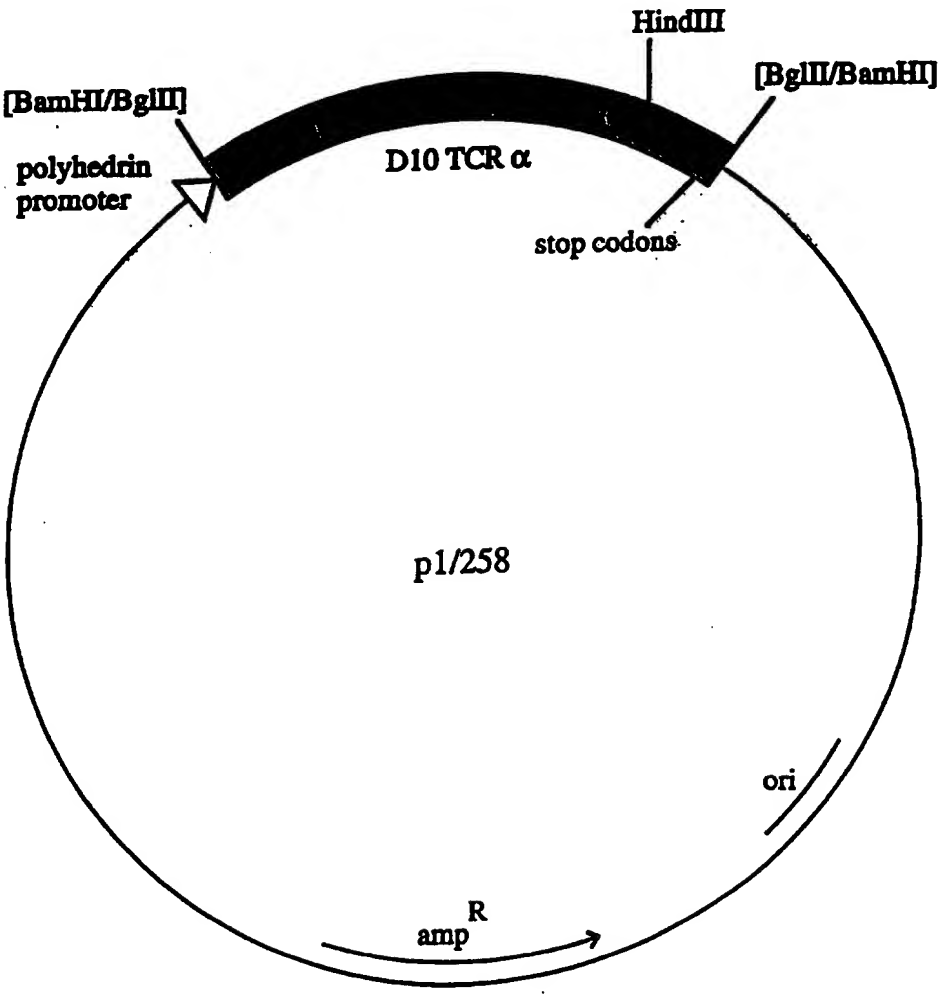

HindIII
[BglII/BamHI]
[BamHI/BglII]
polyhedrin
promoter
D10 TCR α
stop codons
p1/258
ori
R
amp

**FIGURE 21**

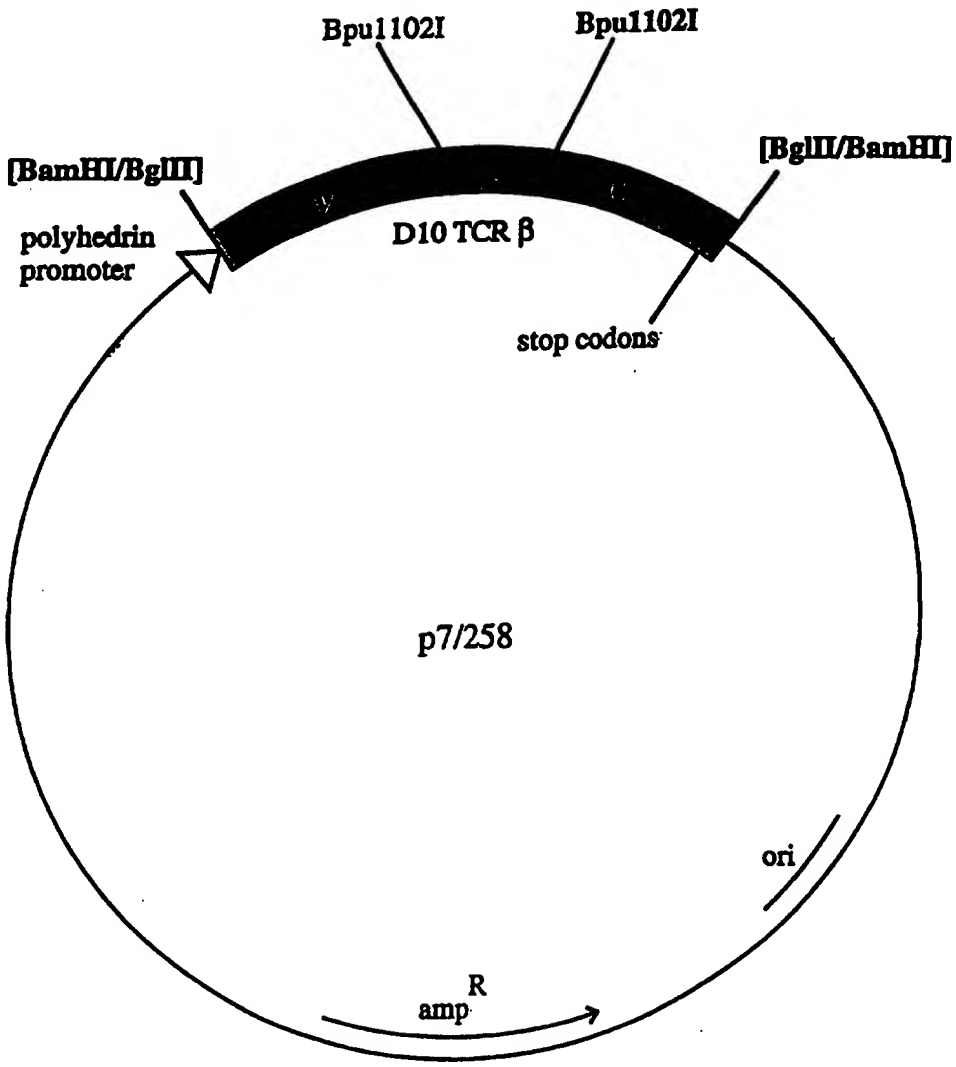

Bpu1102I
Bpu1102I
[BamHI/BglII]
[BglII/BamHI]
polyhedrin
promoter
D10 TCR β
stop codons
p7/258
ori
R
amp

**FIGURE 22**

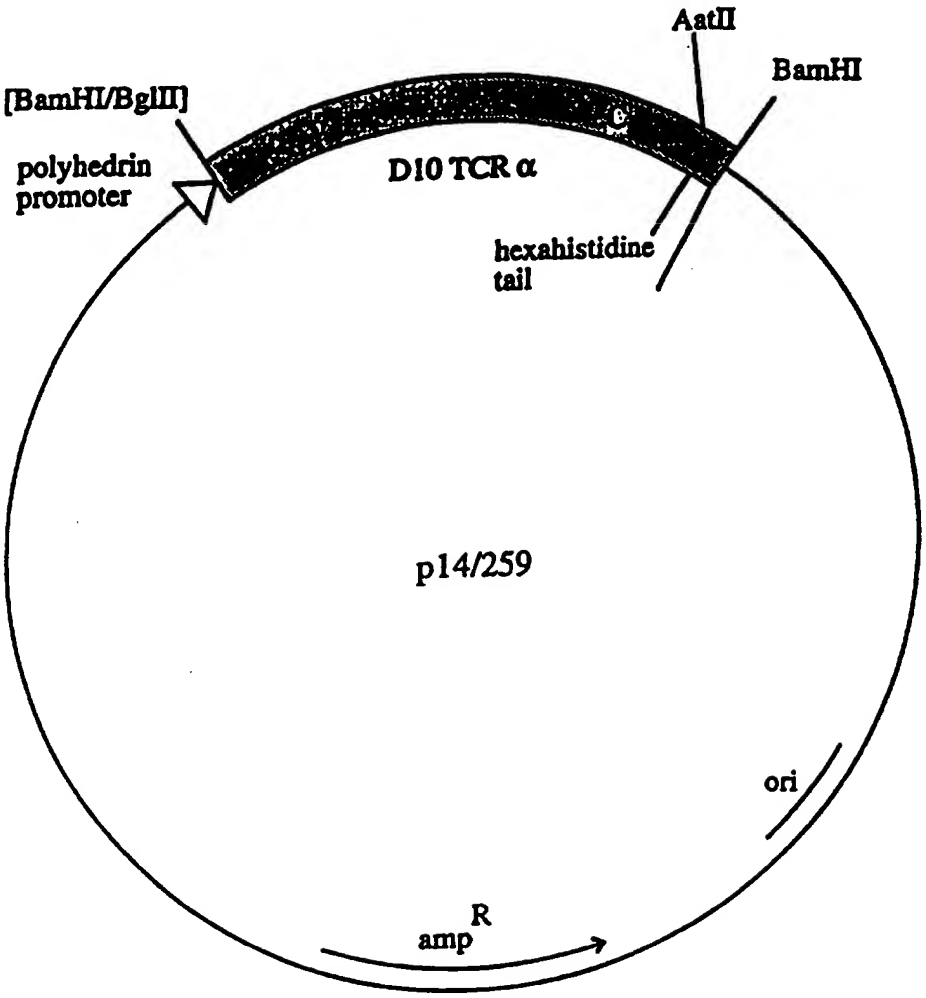

AatII
BamHI
[BamHI/BglII]
polyhedrin
promoter
D10 TCR α
hexahistidine
tail
p14/259
ori
amp R

**FIGURE 23**

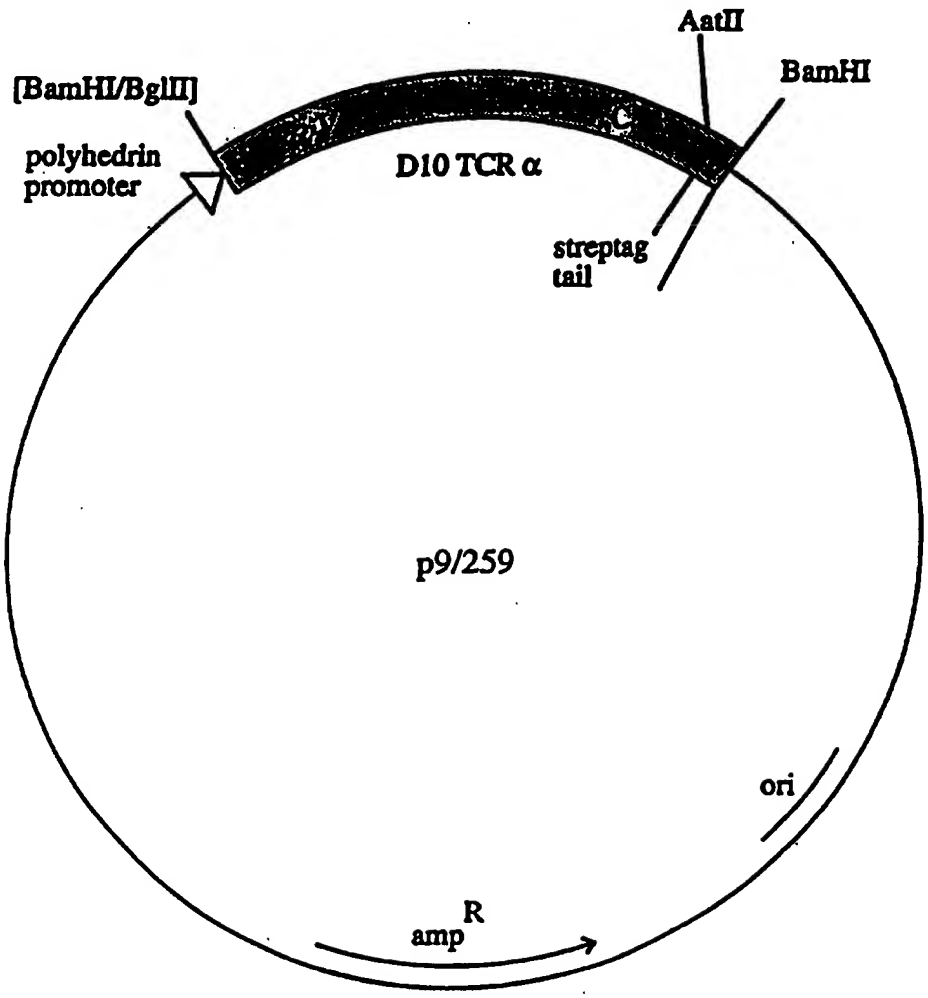
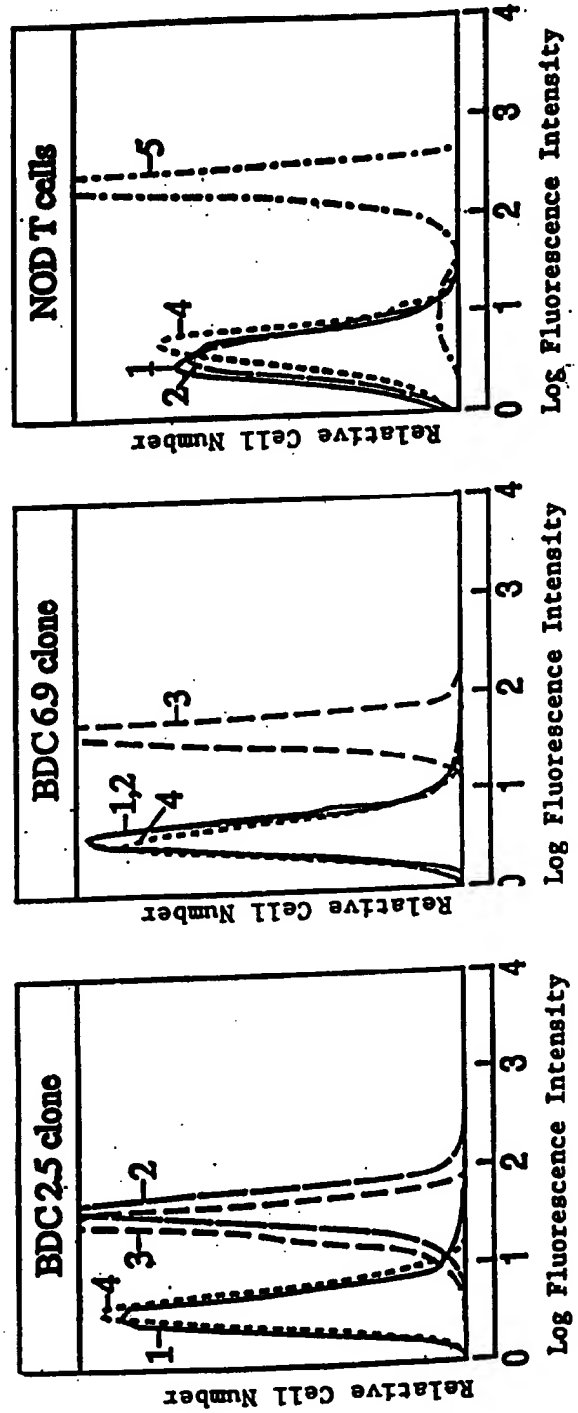
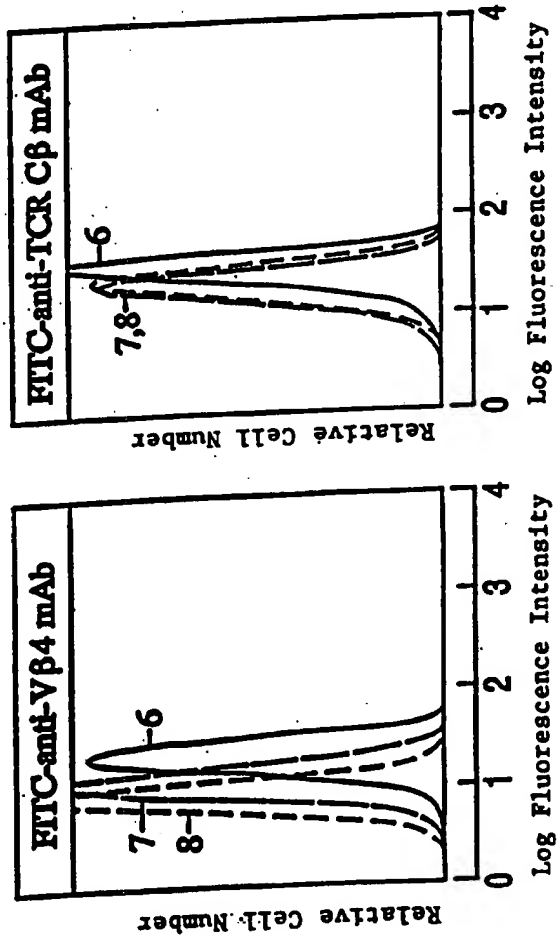

AatII
BamHI
[BamHI/BglII]
polyhedrin
promoter
D10 TCR α
streptag
tail
p9/259
ori
amp R

**FIGURE 24**

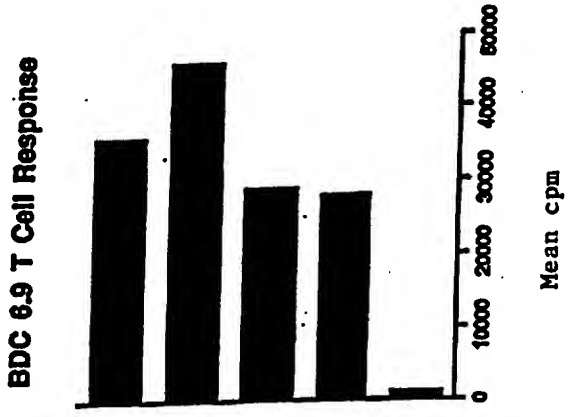
# FIGURE 25A



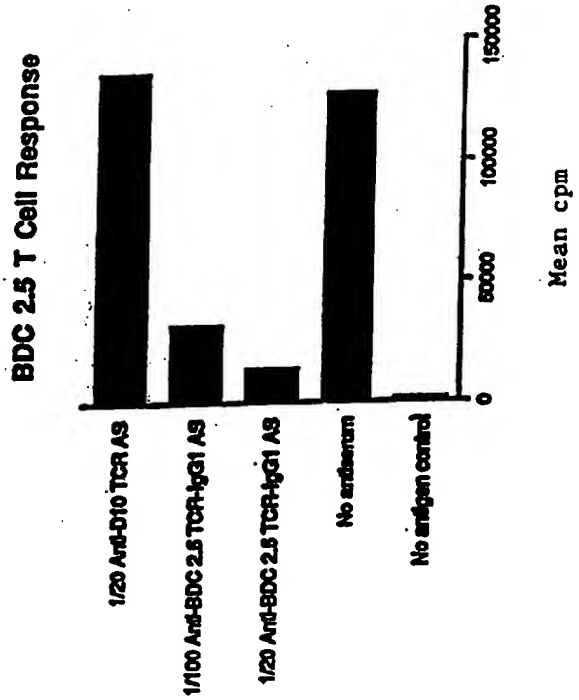

# FIGURE 25B



# FIGURE 26A




# FIGURE 26B



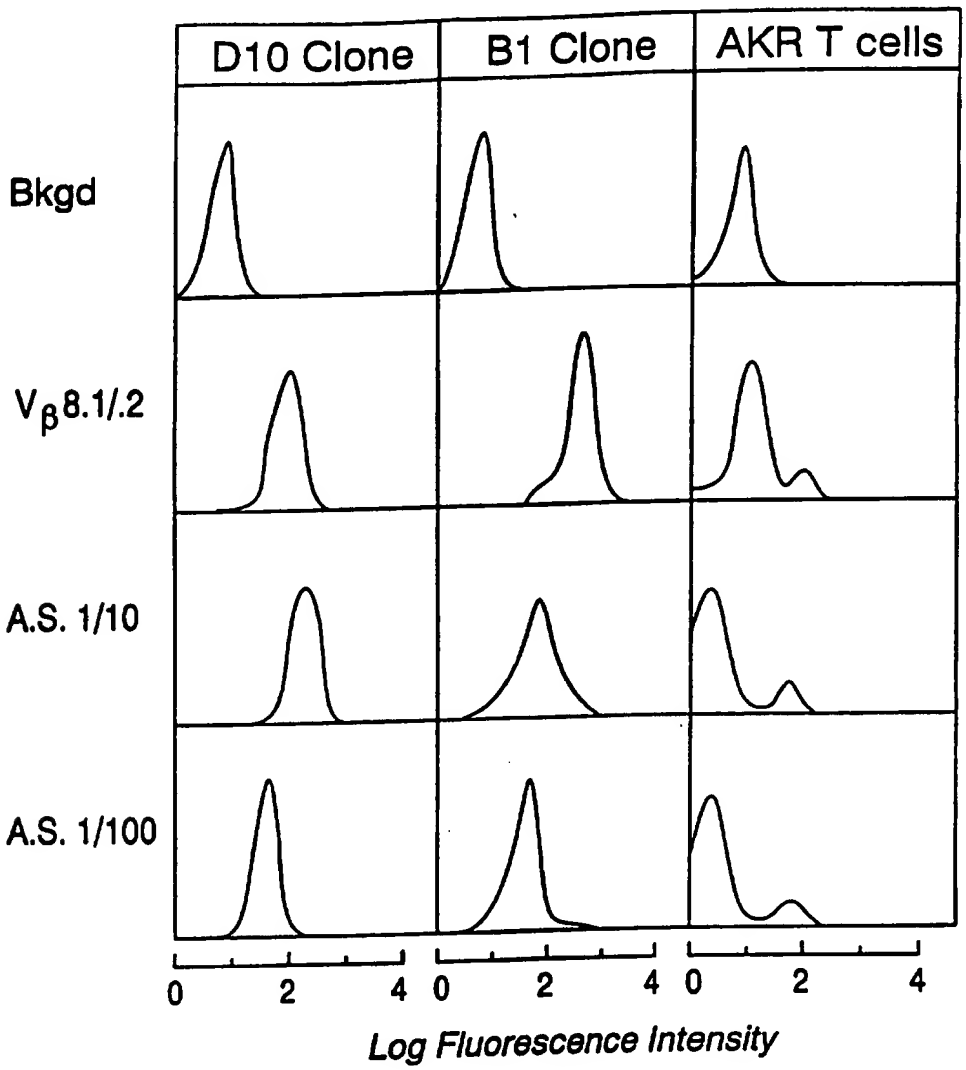

D10 Clone
B1 Clone
AKR T cells
Bkgd
$V_\beta$8.1/.2
A.S. 1/10
A.S. 1/100
0 2 4 0 2 4 0 2 4
Log Fluorescence Intensity

**FIGURE 27**